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Increased expression of hepatocyte growth factor (HGF) and its receptor, Met, has been identified as a possible independent indicator of recurrence in breast cancer patients. Dr. Elliott's laboratory has previously shown increased expression of HGF and Met in regions of invasive human breast cancer. In addition, we have found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met but not HGF. Together, these results suggest that establishment of an autocrine HGF loop in carcinoma cells, and the change in transcriptional and post-transcriptional control of HGF expression is an important indicator of breast cancer progression. Therefore the objectives of my project are a) to examine the transcriptional and post-transcriptional control of HGF expression, and b) to determine the role of signalling molecules in HGF expression in breast carcinoma cells. I have shown that HGF is expressed in many breast carcinoma cell lines and in one particular cell line MCF10A1T3B additional forms of HGF are found. These additional HGF forms are likely the results of proteolytic activity and may represent one mechanism by which the breast carcinoma cells regulate the pericellular level of mature HGF. In addition, I have identified 2 regions in the *HGF* promoter that are responsive to increased c-Src kinase activity. I have shown previously that c-Src kinase is activated in a mouse breast carcinoma cell line, SP1, and c-Src kinase activity is required for HGF-induced motility and anchorage-independent growth of these cells. Expression of an activated form of c-Src (Y527F) increases transcription from the *HGF* promoter and co-expression of Stat3 transcription factor synergistically increases *HGF* promoter activity. These results suggest that c-Src tyrosine kinase activity regulates HGF expression and may be important in the establishment of an HGF autocrine loop in breast carcinoma cells.

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Wesley H June 30, 99

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Introduction

HGF, Met, c-Src and Breast Cancer Progression

Hepatocyte growth factor (HGF) is a multifunctional cytokine. Through binding to its receptor (Met), HGF can induce cell growth (1), differentiation (2) and motility (3). It has been shown that both HGF and Met are essential to embryo development. Mice lacking HGF expression result in impaired placenta, and lethality before birth (4) while homozygous deletion of the *met* gene causes under-development of the limb buds and intra-uterine death (5). Increased expression of HGF and Met has also been identified as a possible independent indicator of recurrence in breast cancer patients (6). Our laboratory has previously shown increased expression of HGF and Met in regions of invasive human breast cancer (7). In addition, we have found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met but not HGF. HGF also stimulates anchorage-independent survival of carcinoma cells (8). Together, these results suggest that establishment of an autocrine HGF loop in carcinoma cells may promote mammary tumor progression.

A number of signalling molecules, such as c-Src (9), Ras(10), Grb2 (11) and phosphatidylinositol (PI) 3-kinase(12), have been shown to be part of the HGF/Met signalling pathway. Activation of Met through binding of HGF, causes auto-phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor tyrosine kinase(13; 14). These tyrosine phosphorylations recruit cytoplasmic signalling molecules and cause their activation. The c-Src non-receptor tyrosine kinase is expressed in many cell types, and its activity is activated in response to HGF and binding to Met (8). Activation of c-Src kinase can lead to increased expression of many genes, including growth factors such as vascular endothelial growth factor (VEGF) (15) and parathyroid hormone-related peptide (16). Increased activation of the tyrosine kinase c-Src occurs in many cancer cells, and c-Src plays a critical role in breast cancer. In a transgenic mouse model, over-expression of an activated form of c-Src induces mammary hyperplasia (17). Furthermore, c-Src kinase is activated during polyoma middle T- induced mammary tumorigenesis in transgenic mice. However, expression of polyoma middle T in *c-src*$^{-/-}$ background mice fails to cause tumor formation(18). Together, these results show that c-Src is necessary, but not sufficient, for mammary tumorigenesis.

HGF Structure, Maturation, and Isoforms

Mature HGF protein contains a heparin binding domain, followed by 4 kringle-like domains designated K1 to K4, as well as a C-terminal region homologous to serine protease but lacking any protease activity (19). In human, HGF is first produced as a single polypeptide precursor of 728 amino acids. Following removal of the N-terminal 31 amino acids, this pro-hormone form is secreted and then cleaved by an unknown serine protease to form the active mature HGF (20). The cleavage at the Arg^{494}-Val^{495} site is essential for HGF activity and several serine proteases have been shown to process pro-HGF at this site *in vitro* (21). Other naturally occurring forms of HGF are also found *in vivo*. Truncated versions of HGF derived from alternative mRNA splicing can act either antagonistically or agonistically towards native HGF. NK1 which contains all the N-terminal amino acids up to K1 can bind to Met and acts in both agonistic or antagonistic manners to HGF depending on culture conditions (22-25). NK2, containing the HGF sequence up to K2, acts mainly in an antagonistic manner (23). The presence of these isoforms in nature may provide a subtle way of regulating HGF activity in vivo.

Hypothesis and Objectives

The overall hypothesis of this laboratory is that HGF is a critical survival factor in breast carcinoma cells during metastasis. Specifically, establishment of an HGF autocrine loop in breast carcinoma cells is

pivotal in the transition from non-malignant to malignant epithelial growth. The change in transcriptional and post-transcriptional control of HGF expression would therefore be an important indicator of breast cancer progression. Moreover, by understanding the transcriptional and post-transcriptional control of HGF expression, therapeutic treatment based on these approaches may be developed towards breast cancer progression and metastasis. The main objectives of my projects are:

1) To examine the transcriptional and post-transcriptional regulation of HGF expression in breast carcinoma cells; and
2) To determine the role of signalling molecules such as c-Src in HGF expression in breast carcinoma cells.

Progress and Results

Objective 1

A) Transcriptional Control

To determine the expression of HGF under the effect of different cytokines, I extracted total RNA from SP1 cells exposed to various cytokines and performed reverse transcription coupled polymerase chain reaction (RT-PCR) to quantitate the amount of *HGF* mRNA in the samples. Primers were designed in Dr. Elliott's laboratory to amplify specifically mouse and human HGF but not its homologue MSP (26; 27) (Figure 1). Primers specific for a constitutively expressed gene β-glucuronidase (GUS B) (28) were used as an internal control. For reliable quantitation, I performed control experiments to determine the optimal conditions for linear amplification in PCR so that the amount of amplification is proportional to the starting amount of mRNA in the sample. As shown in Figure 2 (top panel), PCR was performed with ^{32}P labelled HGF and GUS B primers to amplify cDNA generated from SP1 total RNA with varying number of PCR cycles. The amount of ^{32}P incorporation was determined and plotted against cycle number. It was shown that a linear range of amplification of HGF and GUS B PCR products was achieved between cycles 20 to 30. Next, the effect of template concentration was determined. Serially diluted cDNA was used for PCR with HGF and GUS B primers at 25 cycles. Figure 2 (bottom panel) showed that there is a linear relationship between template dilution and the amount of PCR product generated. Therefore we can semi-quantitatively assess HGF expression in a wide range of template concentrations under the described conditions.

Total RNA from SP1 cells treated with various cytokines was used to synthesize cDNA using AMV reverse transcriptase. Following reverse transcription, one tenth of the reaction was subjected to multiplex PCR with ^{32}P labelled HGF and GUS B primers using the conditions determined above. The amount of HGF expression was normalized to GUS B expression. Figure 3 shows the results of such an analysis. EGF and TGF-β1 have different effects on *HGF* mRNA expression in SP1 cells. Addition of EGF led to an increase of HGF mRNA while TGF-β1 decreased *HGF* mRNA level in SP1 cells. This is in accordance to previous work using fibroblast cell lines (29; 30). Studies with other cytokines such as TNFα and estrogens are in progress.

B)Post-Transcriptional Control

To study HGF expression at the protein level, we developed and characterized two antibodies specific for the N-terminal portion of HGF, and we also characterized an antibody against full length HGF (provided by Genentech). Figure 4A shows the specificity of these 3 antibodies using western blotting analysis. The Genentech antibody was immunoreactive to both α and β chains of HGF on a reducing SDS PAGE gel, while HGF1 and HGF2 antibodies only recognized the α chain. Furthermore to aid the studies of HGF protein, I

used a copper (II) affinity chromatography technique to isolate HGF from conditioned media of cell lines. This technique was previously developed in Dr. Elliott's laboratory (31). Here I have shown that all of the HGF protein bound to the copper (II) column was eluted with 80 mM imidazole in fraction 2 (Figure 4B).

In collaboration with Mr. Jin Gui (Ph.D. student in Dr. Elliott's laboratory) we examined the expression and secretion of HGF from various breast carcinoma cell lines. *HGF* mRNA expression was determined by RT-PCR and protein was purified from conditioned media of these cells using copper (II) affinity chromatography and the protein was detected by western blotting (Figure 5A). All (11/11) mammary epithelial and carcinoma cell lines tested expressed *Met* mRNA and all but one carcinoma cell line (WO-E) expressed met protein. In addition, *HGF* mRNA was expressed in 8/9 cell lines tested and 5/9 expressed HGF protein. The results are summarized in Table I. We have also collaborated on testing HGF expression in non-small cell lung carcinomas (NSCLC), summarized in Table II.

Conditioned media (CM) collected from various breast carcinoma cell lines were tested for the presence of functional HGF using a Met^+ HGF^- cell line, A549 (Figure 5B). In the absence of exogenous HGF, Met protein from A549 cells is not phosphorylated on tyrosine residues. Upon addition of exogenous HGF or CM containing secreted HGF from EL-E, MCF10A1 and MCF10A1T3B, Met protein acquires tyrosine phosphorylation. In contrast, CM from WO-E , which showed no HGF protein had no effect. In CM from one particular breast carcinoma line, MCF10A1T3B, there are several immunoreactive bands (Figure 5A). These additional bands are not seen in the corresponding non-malignant cell line, MCF10A1 or in another breast carcinoma line, EL-E. Interestingly, CM collected from MCF10A1T3B induces Met tyrosine phosphorylation in A549 cells to a lower extent than that in EL-E, suggesting that there is less active HGF in the CM. In the CM from MCF10A1T3B cells, besides the mature form, there also appears to be unprocessed pro-HGF at 90 kDa and two smaller bands at 56 kDa and 32 kDa. The latter form may be NK2, which has a M_r of approximately 32 kDa; the structure of the larger form is unknown.

Further experiments showed that these smaller forms of HGF are derived from degradation of mature HGF (Figure 6). Incubation of recombinant HGF with MCF10A1T3B cells resulted in the appearance of the 56 kDa and 32 kDa forms, whereas incubation with Cos-1 cells has no effect on the integrity of the recombinant HGF. This result suggests that the two additional HGF bands arise from proteolytic activity associated with MCF10A1T3B cells. Together these data suggests that the additional forms of HGF observed in CM from MCF10A1T3B cells represent one mechanism by which the breast carcinoma cells regulate the pericellular level of mature HGF, and thereby modulate HGF-dependent cell function *in vivo*.

Further experiments are underway to determine the nature of the protease(s) responsible for the degradation, and the structures and functions of these different forms of HGF in MCF10A1T3B CM.

Objective 2

We have used a mouse mammary carcinoma cell line, SP1, for studies of HGF expression and function. Dr. Elliott's laboratory has previously shown that SP1 cells express HGF and tyrosine phosphorylated Met, suggesting a possible autocrine loop (32). I have shown that c-Src kinase is activated in SP1 cells (8). Furthermore, c-Src kinase activity is required for HGF-induced motility and anchorage-independent growth of these cells (8). Since c-Src has been reported to regulate expression of some growth factors, I have examined whether elevated c-Src kinase activity promotes the establishment of an HGF autocrine loop in breast carcinoma cells. Therefore, I studied the role of c-Src in regulating HGF mRNA and protein expression. The tyrosine kinase inhibitor herbimycin A (33; 34) causes a two-fold reduction in HGF mRNA in SP1 cells, while the phosphatidyl inositol (PI) 3-kinase inhibitor LY294002 (35) shows no effect (Figure 7). In addition, expression of a dominant negative mutant of c-Src (K295R, Y527F) (36) in SP1 cells leads to similar levels of reduction in HGF mRNA (Figure 8A) and functional protein (Figure 8B) comparable to herbimycin A treatment. These findings imply that c-Src kinase is the major tyrosine kinase regulating HGF expression.

Transient expression studies in both SP1 and Cos-1 cells further support the notion that c-Src kinase activity is required for *HGF* transcription. Using a reporter construct consisting of the full length *HGF* promoter (2.7 kb) linked to the luciferase gene, I studied the effect of c-Src kinase activity on *HGF* promoter-directed luciferase expression in SP1 and Cos-1 cells in transient transfection studies. Expression of activated c-Src (SRC F527) (37) induces *HGF* promoter activity while expression of the dominant negative c-Src (SRC RF) reduces *HGF* promoter activity (Figure 9, top panels). There is a corresponding change in c-Src kinase activity in the cells transfected with different mutant forms of c-Src (Figure 9, bottom panel). To further understand the mechanism of regulation of HGF expression by c-Src kinase, I constructed a series of deletion mutants of the *HGF* promoter fused to luciferase and transiently transfected these into Cos-1 cells and also co-expressing activated or dominant negative forms of c-Src (Figure 10). Using this approach, I have located two regions responsive to increased c-Src kinase activity. Deletion of the 5' region -1231 to -755 of the *HGF* promoter resulted in a loss of *HGF* promoter activity in response to activated c-Src (approximately one-third less than that of full length promoter). Deletion of this region and another region between -274 and -70 eliminated all the response of *HGF* promoter to activated c-Src, suggesting that both of these regions are important in c-Src mediated *HGF* transcription. Both of these regions contain putative binding sites for Stat3 protein (Figure 11). Stat3 has been shown to activate transcription in response to increased c-Src kinase activity (38-41). In breast development, Stat3 is activated by c-Src leading to tubule formation (42). Therefore, I investigated whether Stat3 is involved in the regulation of HGF expression in response to c-Src. Co-expression of activated c-Src with Stat3 can synergistically increase transcription from the *HGF* promoter, suggesting that Stat3 is part of the signalling pathway that regulates HGF expression (Figure 12A).

Integrin-linked kinase (ILK) is a serine/threonine kinase that interacts with integrins β1, β2, and β3, and its activity is regulated by the presence of extracellular matrix proteins (43). Overexpression of ILK in epithelial cells can induce tumor formation in nude mice and also allow anchorage-independent growth of cells (a requirement for metastasis) (44; 45). Activity of ILK has been shown to regulate expression of various cell cycle genes, such as p21 and p27 cyclin-dependent kinase inhibitors (44). Activation of ILK leads to increased expression of Lef-1 transcription factor, which complexes with β-catenin to activate transcription (46). I have found that expression of dominant negative ILK significantly reduces *HGF* transcription while expression of the wildtype form has little or no effect (Figure 12B). Since there are several Lef-1 binding sites on the HGF promoter (Figure 11), it is possible that ILK regulates HGF transcription through Lef-1. ILK may regulate HGF expression in cells allowing them to survive anchorage-independent growth during tumor progression. Further studies are in progress to clarify the roles of ILK and Lef-1 in regulating HGF expression under different anchorage conditions.

Conclusions

In summary, I have initiated RT-PCR studies of HGF expression in SP1 breast carcinoma cells. The effects of EGF and TGF β1 were examined and further studies are in progress to examine the effect of other cytokines. In collaboration with Mr. Jin Gui, I have examined HGF expression of many breast carcinoma cell lines. Most lines express *HGF* mRNA but only a few cell lines secrete functional HGF protein. In one breast carcinoma cell line MCF10A1T3B, two smaller forms of HGF are detected and they are likely to be products of protein degradation of mature HGF. It has been shown that tumor cells can affect gene expression of the surrounding non-malignant stromal cells. Furthermore, the naturally occurring isoform of HGF, NK2, has been shown to act as an antagonist to native HGF. Therefore, these new forms of HGF may be an important way by which breast carcinoma cells regulate their HGF responsiveness or the growth and gene expression of their surrounding non-malignant cells. The next stage of studies will involve identifying the structures and functions of these forms of HGF and the protease(s) responsible for their production. The additional forms of HGF in

CM from MCF10A1T3B cells will be purified using HPLC and other chromatographic techniques. Once purified the proteins will be partially sequenced to determine the structures, and will be used in functional assays (proliferation, motility and Met tyrosine phosphorylations assays) to determine their functions. Protease(s) responsible for the generation of these additional HGF isoforms will be isolated using affinity chromatographic techniques. Their expression and substrate specificity will also be studied.

Studies of *HGF* transcription have revealed that HGF expression is under the regulation of c-Src tyrosine kinase. c-Src kinase has been shown to regulate the expression of other growth factors such as VEGF. In the case of VEGF, c-Src regulates expression through the transcription factor HIF-1 (15). I have identified 2 regions on the *HGF* promoter that are responsive to increased c-Src kinase activity. The pathway by which c-Src kinase regulates HGF expression may involve the transcription factor, Stat3. Co-expression studies confirms that Stat3 can enhance the c-Src responsiveness of the *HGF* promoter. Electrophoretic mobility shift assays, footprinting assays and site-directed mutagenesis of Stat3 sites in the *HGF* promoter will reveal how Stat3 regulates *HGF* transcription.

Furthermore, ILK a serine/threonine that regulates cell survival in anchorage-independent conditions may also be involved in the regulation of HGF expression. Expression of the dominant negative form of ILK leads to decreased transcription from the *HGF* promoter. The presence of putative Lef-1 binding sites on the *HGF* promoter suggests that ILK may regulate HGF expression through Lef-1. The relationship between ILK kinase activity, anchorage-independent growth and HGF expression is currently being investigated.

Finally to study the role of HGF expression in breast cancer progression *in vivo*, I am planning to construct a transgenic mouse strain carrying a reporter gene (β-galactosidase) fused to the HGF promoter. This mouse strain will be crossed with another transgenic mouse strain that harbours the MMTV LTR-driven expression of polyoma middle T protein (47). Expression of polyoma middle T protein under these conditions will be limited to breast epithelial cells and therefore, will induce breast tumors (48; 49). Expression of HGF can be monitored by the expression of β-galactosidase as breast tumor progresses in the transgenic mice. This will reveal the stage of breast tumor progression at which HGF expression is up-regulated.

These studies will provide insight to the de-regulation of HGF expression in breast carcinoma cells and may provide novel targets for treatment.

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TABLE I

Expression of HGF and Met in human breast epithelial and carcinoma cell lines

Breast Cell Line	Malignant Status	HGF			Met	
		mRNA	Protein	Activity	mRNAA	Protein
WO-E [a]	Yes	-[c]	-	-	+	-
EL-E [a]	Yes	+	+	+	+	+
MCF10A1[b]	No	+	+	+/-	+	+
MCF10A1.T3B[b]	Yes	+	+	+/-	+	+

Legend:

a) **EL-E and WO-E are human breast carcinoma cell lines derived from human breast cancer patients (obtained from Dr. B. Campling, Cancer Research Lab., Queen's University).**

b) **MCF10A1 is a subclone of a spontaneously immortalized non-tumorigenic human breast epithelial cell line established from long term culture of a breast subcutaneous mastectomy. MCF10A1.T3B is a cell line derived from *Ha-Ras* transfected MCF10A1 cells growing as a tumor in a nude mouse (obtained from Dr. F. Miller, Michigan Cancer Foundation).**

c) **+, positive; -, negative; +/- trace amount**

TABLE II

Expression of HGF and Met in human Non-small cell lung carcinoma cell lines

Non Small Cell Lung Carcinoma Lines [a]	Histology	Origin	HGF			Met		HGF-induced DNA synthesis[c]
			mRNA	Protein	Activity	mRNA	Protein	
SW-900	squamous cell carcinoma	primary tumor	+[b]	+	+	+	+	+
SK-MES-1	squamous cell carcinoma	pleural effusion	+/-	+/-	+	+	+	+/-
WT-E	squamous cell carcinoma	pleural effusion	+/-	+	-	+	+	+
FR-E	large cell anaplastic	pericardial effusion	+/-	+/-	-	+	+/-	-
SK-Luci-6	large cell anaplastic	primary tumor	+	+	+	+	-	-
QU-DB	large cell anaplastic	primary tumor	+/-	-	-	+	-	-
BH-E	adenocarcinoma	pleural effusion	+	+	+	+	-	-
LC-T	adenocarcinoma	primary tumor	-	-	-	+	-	-

Legend:

c) Obtained from Dr. B. Campling (Cancer Research Lab., Queen's University).

d) N/D, not determined; +, positive; +/-, trace amount; -, negative.

e) Cells ($1x10^4$) in triplicate in a 24 well plate were incubated for 24 hours at 37°C and 5% CO_2, alone, or with HGF (20 ng/ml). After 24 hours, ^{3}H-Thymidine was added, and cells were incubated for a second 24 hours period. DNA synthesis was measured as incorporation of ^{3}H-Thymidine.

Figure 2 Determination of Optimal Conditions for Quantitation of HGF Expression by RT-PRC

SP1 cells were lysed with TriZol reagent (Life Technology) and total RNA was extracted according to manufacturer's instructions. cDNA was synthesized from 1 mg of total RNA using AMV reverse transcriptase. The resulting cDNA was used to determine the optimal PCR conditions that would allow linear amplification of HGF and an internal control gene transcript (GUS B).

Top panel: The amount of cDNA was held constant while varying the number of PCR cycles to determine the range of linear amplification. After an initial denaturation cycle at 94°C for 2 min, the reactions went through cycles consisting of 94°C, 1 min, 55°C, 1 min, 72°C, 1 min. PCR reactions were stopped after the indicated number of PCR cycle was completed, and was analyzed by agarose gel electrophoresis. Bands corresponding to HGF and GUS B products were excised and the amount of ^{32}P incorporation was determined by scintillation counting technique.

Bottom panel: Effect of cDNA concentration was studied by serially diluting template in a PCR reaction with 5' ^{32}P end-labelled HGF and GUS B specific primers. 25 cycles of (94°C, 1 min, 55°C, 1 min, 72°C, 1 min) was done. Determination of ^{32}P incorporation into the PCR products were as described above.

Figure 3 Effect of growth factors on HGF mRNA expression in SP1 Cells

SP1 cells were plated at 80% confluence and prestarved overnight. Cells were then treated with either 1 μM EGF or 1 ng/ml TGFβ-1. At indicated time point, cells were lysed with TriZol reagent. Total RNA was extracted, and cDNA was synthesized as described in Figure 1. One tenth of the reaction was used for multiplex PCR analysis using 5' end-labelled HGF and GUSB (internal control) specific primers at conditions optimized for linear quantitation of GUSB and HGF. After 25 cycles of (94°C, 1 min, 55°C, 1 min, 72°C, 1 min), PCR reactions were analyzed by gel electrophoresis on a 2% agarose gel. The bands corresponding to HGF and GUSB PCR products were excised and counted by scintillation counting technique. The amount of HGF mRNA was normalized to the amount of GUS B mRNA in each sample. The amount of HGF mRNA expression compared to control was plotted and is shown.

Figure 4

Panel A: Characterization of anti-HGF antibodies.

Recombinant HGF was denatured in reducing or non-reducing SDS sample buffer before fractionated on 8% SDS PAGE gel. Following electro-transfer of proteins onto nitrocellulose, the blots were probed with either Genentech sheep anti-HGF antibody or our rabbit anti-HGF antibody (HGF1 and HGF2). The positions of mature HGF, α and β chains are indicated.

Panel B: Purification of HGF using Copper (II) affinity column

100 ng of recombinant HGF was diluted in equilibration buffer (20 mM sodium phosphate, pH 7.4, 1 M NaCl, 1 mM imidazole) and loaded onto a Pharmacia HiTrap column charged with copper ions. One 1 ml fraction was collected after loading the column and was designated as flow-through fraction (FT). The column was washed with 15 column volume of equilibration buffer before eluted with equilibration containing 80 mM imidazole. Five 1 ml fractions were collected and the column was stripped with equilibration buffer containing 5 mM EDTA. All of the copper ions came out in first 2 ml of "stripping" (S1 and S2). All fractions were concentrated in microcon (Amicon) before analyzed on reducing SDS PAGE gel. Following electrophoresis, proteins were transferred onto nitrocellulose and the blot was probed with anti-HGF2 antibody.

Figure 5 Expression and activity of HGF in human breast carcinoma cell lines

Panel A: Breast epithelial cell line MCF10A1 and carcinoma cell lines WO-E, EL-E and MCF10A1T3B were plated at 80% confluence and allowed to adhere to plates. Media were then replaced with serum-free DMEM and allowed to incubate for 24 hours. The conditioned media (CM) were then collected and loaded onto a copper (II) affinity column as described in Figure 4. HGF containing fractions were concentrated, fractionated on a reducing SDS PAGE gel, and followed by western blotting analysis. The positions of pro-HGF and HGF α chain are indicated. Additional HGF isoforms in MCF10A1T3B lane are indicated with short arrows.

Panel B: CM were collected from WO-E, EL-E, MCF10A1 and MCF10A1T3B cell lines, and were added to prestarved A549 adenocarcinoma cells (which express Met but not HGF). Controls consisted of cells incubated with DMEM alone, or with 20 ng/ml HGF. After incubation for 30 minute at 37°C, the cells were washed twice with ice cold PBS, lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% NP-40) containing 1 mM PMSF, 1μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM Na_3VO_4. Equal amount of protein were immunoprecipitated with anti-Met antibody (Santa Cruz), analyzed on 7% SDS PAGE gel, and proteins were transferred to nitrocellulose. The blot was probed with PY-20 anti-phosphotyrosine antibody to determine Met tyrosine phosphorylation level (top panel) and then stripped and re-probed with anti-Met antibody to determine the total amount of Met protein in each sample (bottom panel).

Figure 6 Degradation of recombinant HGF by MCF10A1T3B cells

10^4 MCF10A1T3B or Cos-1 cells were seeded and allowed to adhere overnight before washed with PBS and replaced with serum-free media in the presence or absence of 20 ng recombinant HGF. As controls, 20 ng of recombinant HGF was incubated alone (None) or with 2 ng trypsin in 50 mM Tris (pH 7.4). Following incubation at 37°C for 24 hours, the media were removed and concentrated with microcon and analysed by western blotting analysis using anti-HGF2 antibody.

Figure 7 Effect of Inhibitors on HGF mRNA Expression in SP1 Cells

SP1 cells were plated at 80% confluence and prestarved overnight. Cells were then treated with

either 3 μM LY294002 or 10 nM herbimycin A. At indicated time point, cells were lysed with TriZol reagent and total RNA was extracted. cDNA was synthesized and RT-PCR reactions were performed as described in Figure 2. The amount of HGF mRNA expression compared to control was plotted and is shown.

Figure 8 Effect of dominant negative c-Src kinase expression in SP1 cells on HGF mRNA and function protein levels.

Panel A: Pooled SP1 cells transfected with dominant negative Src (SRC-RF; K295R,Y527F) or wildtype Src (SRC) or untreated SP1 cells were plated and prestarved as in Figure 1. c-Src kinase activity has been shown previously to be reduced in SRC-RF cells (4). Total RNA extraction, RT-PCR analysis were performed as in Figure 1. HGF mRNA expression in each of the cell line is expressed as a % of untransfected control cells..

Panel B: Conditioned media were collected from pooled SP1 cells transfected with SRC-RF, SRC or untreated SP1 cells, and were added to prestarved A549 adenocarcinoma cells to assess the amount of functional HGF in each CM as described in Figure 5. Following SDS PAGE and electro-transfer, the blot was probed with PY-20 anti-phosphotyrosine antibody to determine Met tyrosine phosphorylation level (top panel) and then stripped and re-probed with anti-Met antibody to determine the total amount of Met protein in each sample (bottom panel).

Figure 9 The effect of c-Src kinase mutant expression on total c-Src kinase activity and *HGF* promoter activity

SP1 (panels A and C) and Cos-1 cells (panels B and D) were co-trasnfected with an HGF-luciferase reporter plasmid (2.7 HGF-luc) and expression vectors expressing wildtype c-Src (SRC), activated Src (SRC-F527) or dominant negative Src (SRC-RF) or an empty expression vector (none) as described in Figure 4. After 48 hours, cells were lysed and a portion of the lysate was used for luciferase assays. Luciferase activity was measured as light emission and detected by luminometer. The amount of luciferase activity in each sample was normalized to transfection efficiency as determined by β-galactosidase activity as an internal control. Equal amount of the remaining lysates were used to detect c-Src kinase activity using enolase as a substrate. Results of the luciferase assays are shown in panels A and B and the amount of c-Src kinase activity (normalized to total c-Src protein) is represented graphically in panels C and D.

Figure 10 Mapping of c-Src kinase responsive regions in the *HGF* promoter

Cos-1 cells were co-transfected with HGF-luciferase reporter plasmid (2.7 HGF-luc) or reporter construct containing a deletion of the HGF promoter (1.2, 0.8, 0.5, 0.5 Δ, 0.3 and 0.1) and expression vectors expressing wildtype c-Src (SRC), activated Src (SRC-F527), dominant negative Src (SRC-RF) or empty expression vector (None) as described in Figures 9 and 10.

Panel A: Graphical representation of luciferase activity from each sample.
Panel B: Schematic representation of the HGF deletion mutants used in Panel A.

Figure 11 Proposed Organization of the *HGF* Promoter

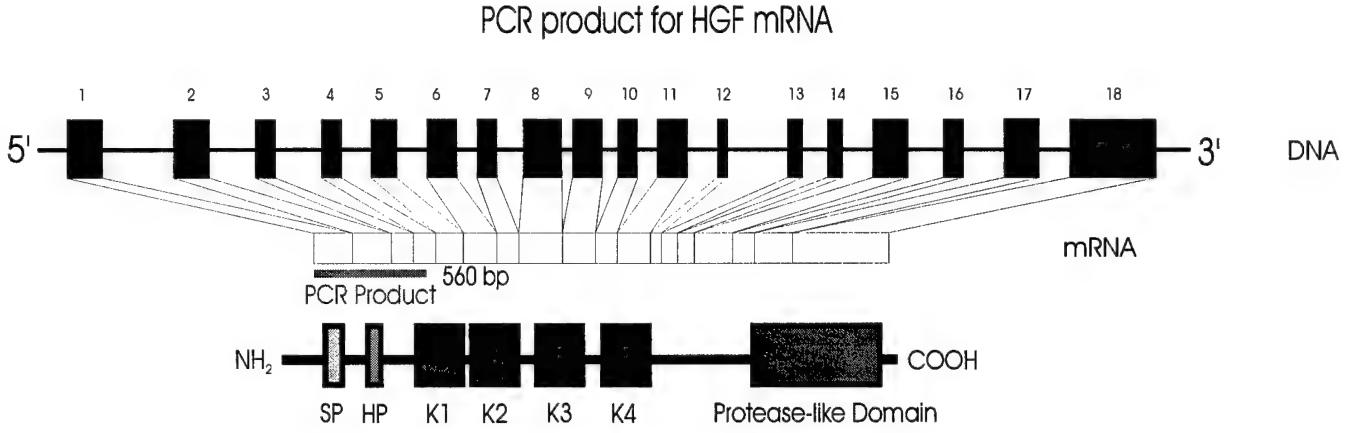
The HGF promoter sequence was scanned for known consensus binding sequence for transcription factors. A number of binding sites were identified and are shown schematically here. Chicken ovalbumin upstream promoter-transcription factor (COUP-TF), estrogen receptor (ER), Sp1 and CAATT/enchancer binding protein (C/EBP) have been shown to affect HGF expression in various cell lines. Consensus sites for ETS, AP-1, TGF-β inhibitory element binding protein (TIE BP), IL-6 responsive element binding protein (IL-6 RE BP), Lef-1 and Stat1, 3, 5 are found but their roles have not been confirmed. Two regions of the HGF promoter that are responsive to c-Src kinase activity are highlighted.

Figure 12 Effect of Stat3 and ILK expression on *HGF* promoter activity.

Panel A: Cos-1 cells were transfected with an HGF-luciferase reporter plasmid (2.7 HGF-luc) and empty expression vector or with vectors expressing activated Src (SRC-F527) in combination with wildtype (Stat3) or dominant negative Stat3 (Stat3β). Transfections and luciferase assays were performed as described in Figures 9 and 10.

Panel B: Cos-1 cells were transfected with 2.7 HGF-luc reporter plasmid and an empty expression vector or with vectors expressing ILK or kinase-dead ILK (ILK KD). A control reporter plasmid containing no HGF promoter sequence was transfected into Cos-1 to determine the baseline luciferase activity level. Transfections and luciferase assays were performed as described in Figures 9 and 10.

A



B

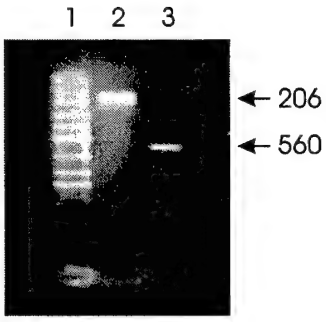


Figure 1:

Panels A: Design of PCR primers for HGF
Primers were designed to overlap more than one exon and to crossreact between mouse and human. HGF primers: 5' (sense) TGT CGC CAT CCC CTA TGC AG (corresp. to bases 69-88 of hHGF); 3' (antisense) TCA ACT TCT GAA CAC TGA GG. (corresp. to bases 610-629 of hHGF).

Panel B: Detection by RT-PCR of HGF mRNA in mouse breast carcinoma cell line, SP1:
cDNA was prepared from 1 μg of total RNA, and subjected to RT-PCR of 25 cycles of 1 min at 95°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (elongation). Lane 1: DNA molecular size markers; lane 2, 206 bp marker; lane 3, PCR product of HGF.

Figure 2

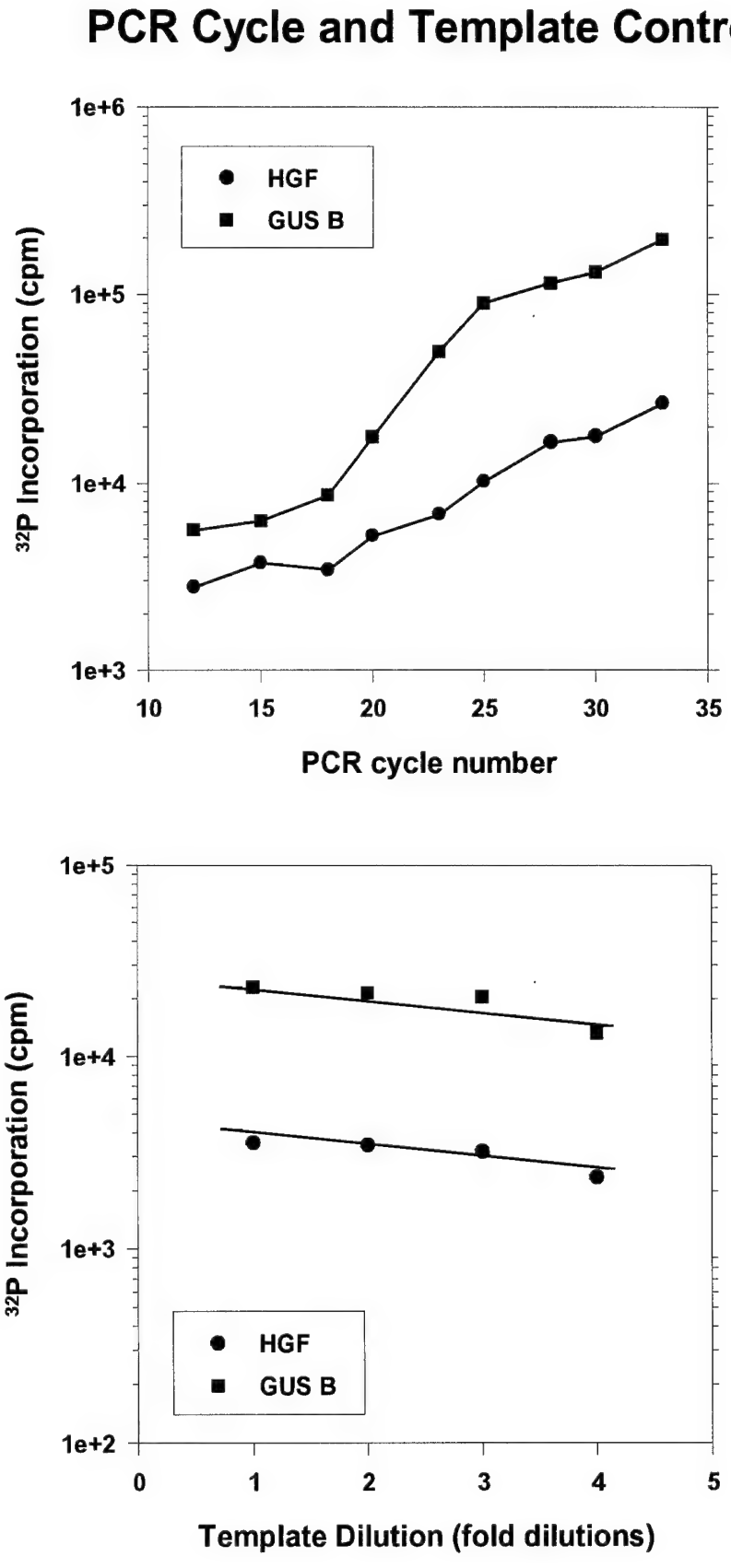

PCR Cycle and Template Control
1e+6
1e+5
1e+4
1e+3
HGF
GUS B
32P Incorporation (cpm)
10
15
20
25
30
35
PCR cycle number
1e+5
1e+4
1e+3
1e+2
HGF
GUS B
32P Incorporation (cpm)
0
1
2
3
4
5
Template Dilution (fold dilutions)

Figure 3

Effect of growth factors on HGF expression in SP1 cells

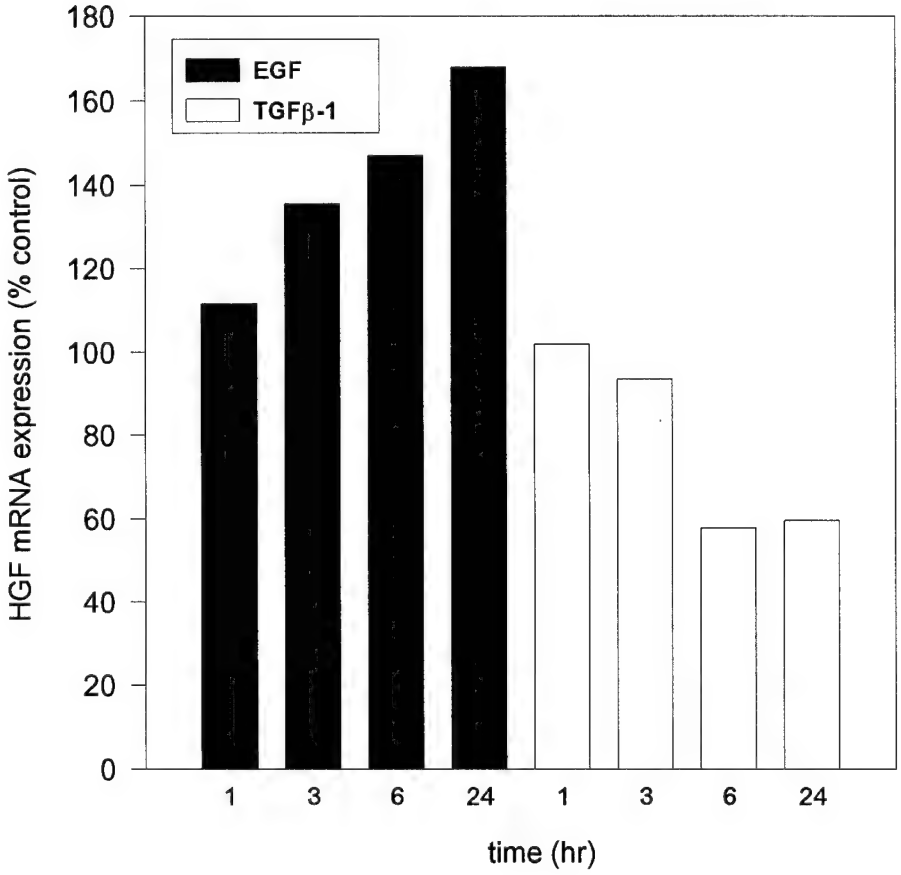
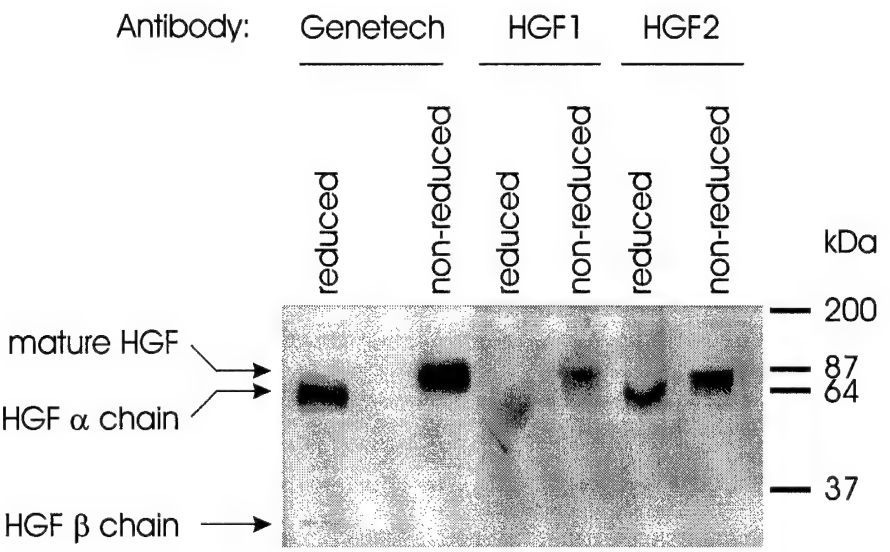


Figure 4

A


Antibody:
Genetech
HGF1
HGF2
reduced
non-reduced
reduced
non-reduced
reduced
non-reduced
kDa
200
87
64
37
mature HGF
HGF α chain
HGF β chain

B

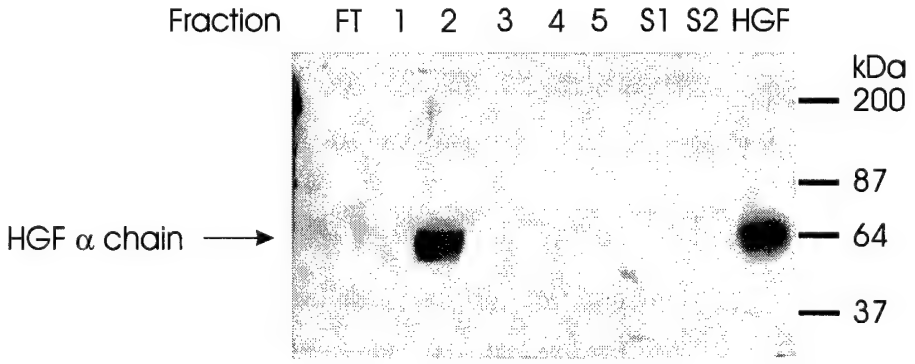

Fraction
FT
1
2
3
4
5
S1
S2
HGF
kDa
200
87
64
37
HGF α chain

Figure 5

Expression and activity of HGF in human breast carcinoma cell lines

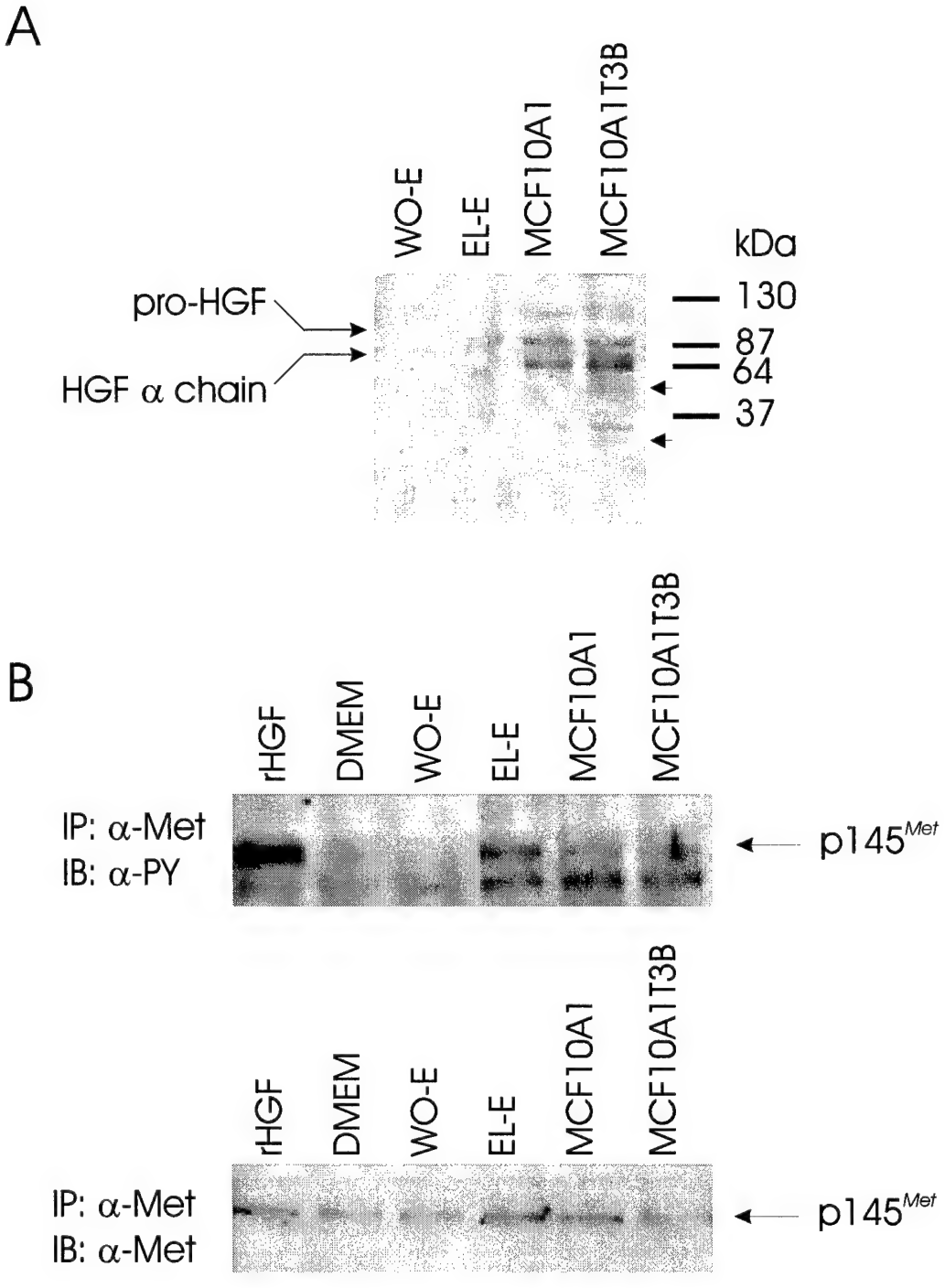


Figure 6

Degradation of recombinant HGF by MCF10A1 T3B Cells

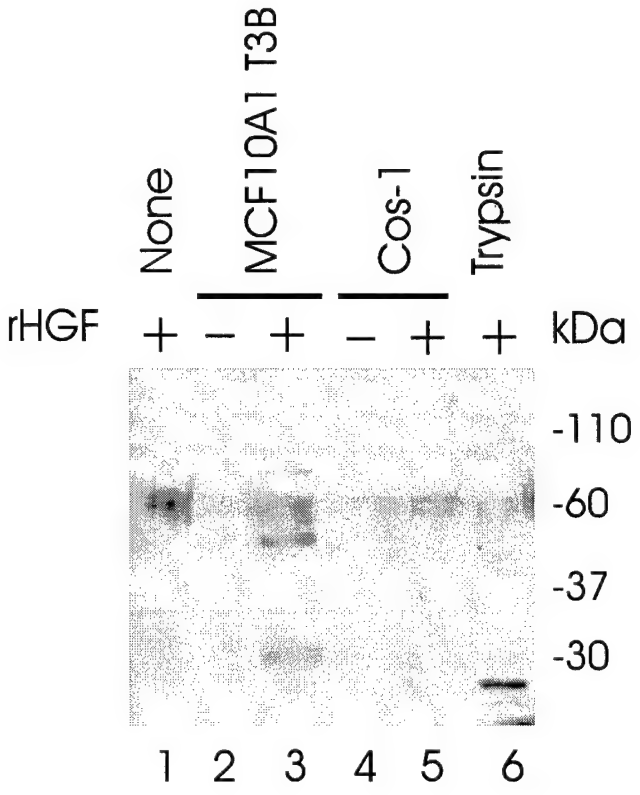


Figure 7

Effect of Signal Transduction Inhibitors on HGF mRNA Expression in SP1 cells

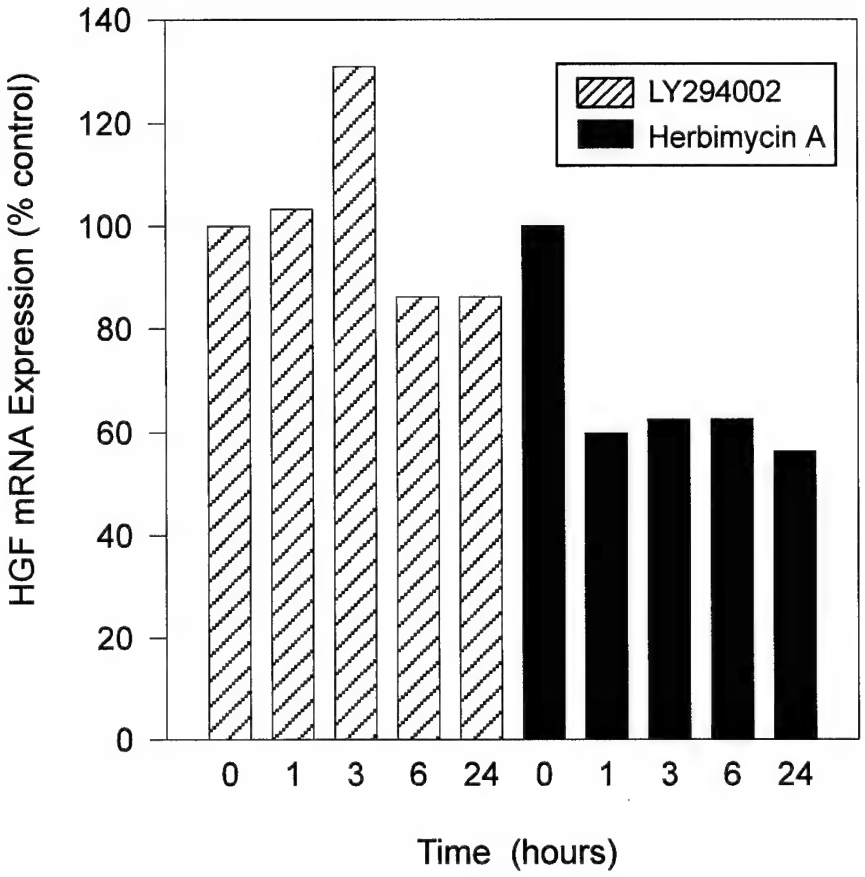
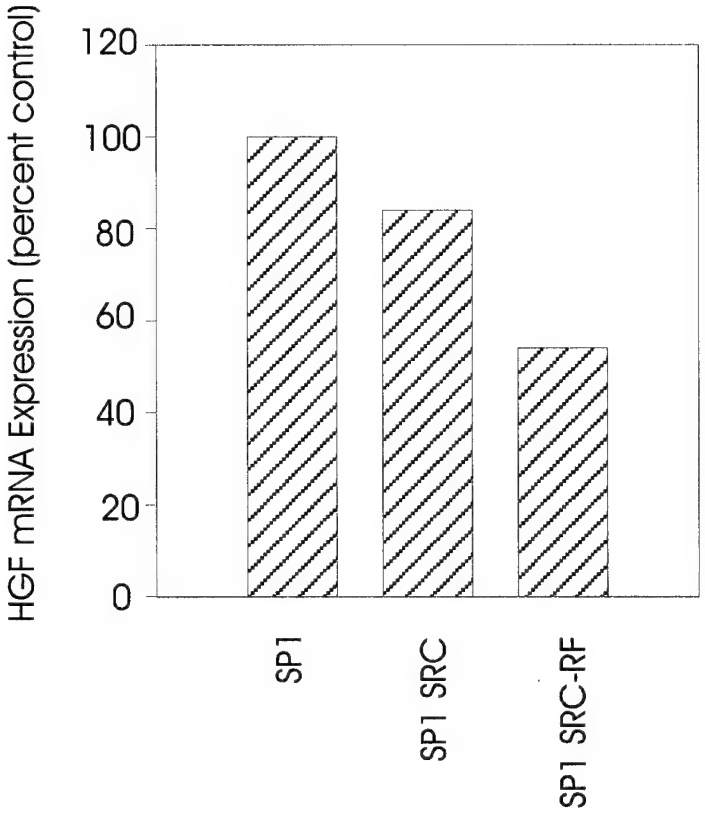
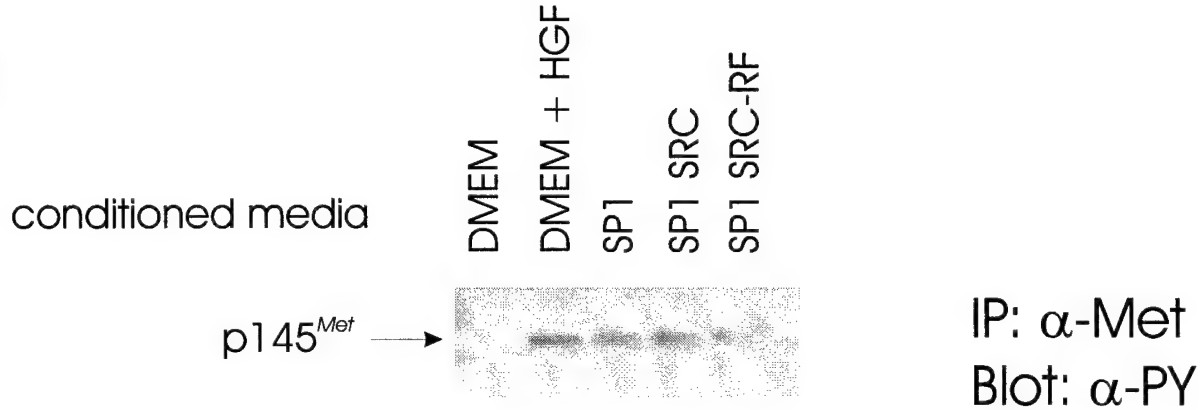


Figure 8

A


HGF mRNA Expression (percent control)
120
100
80
60
40
20
0
SP1
SP1 SRC
SP1 SRC-RF

B


conditioned media
DMEM
DMEM + HGF
SP1
SP1 SRC
SP1 SRC-RF
p145Met
IP: α-Met
Blot: α-PY

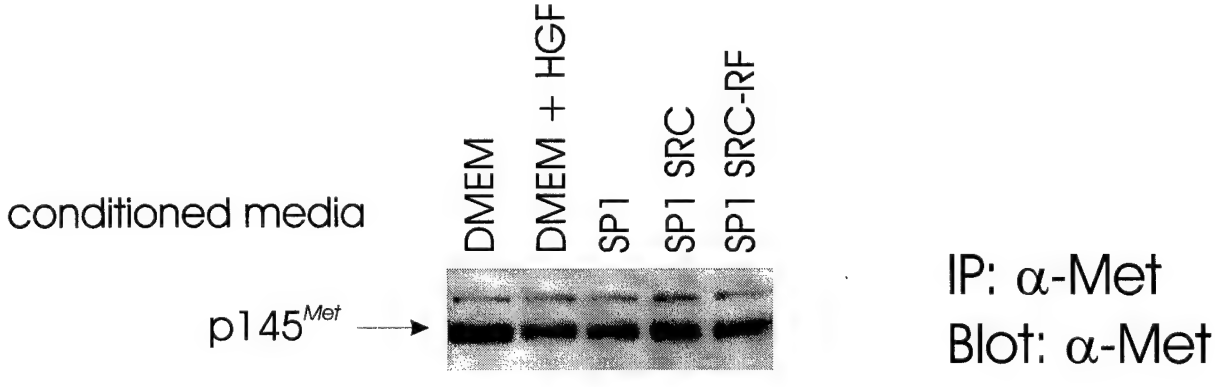

conditioned media
DMEM
DMEM + HGF
SP1
SP1 SRC
SP1 SRC-RF
p145Met
IP: α-Met
Blot: α-Met

Figure 9

The effect of c-Src kinase mutant expression on total c-Src kinase activity and *HGF* promoter activity

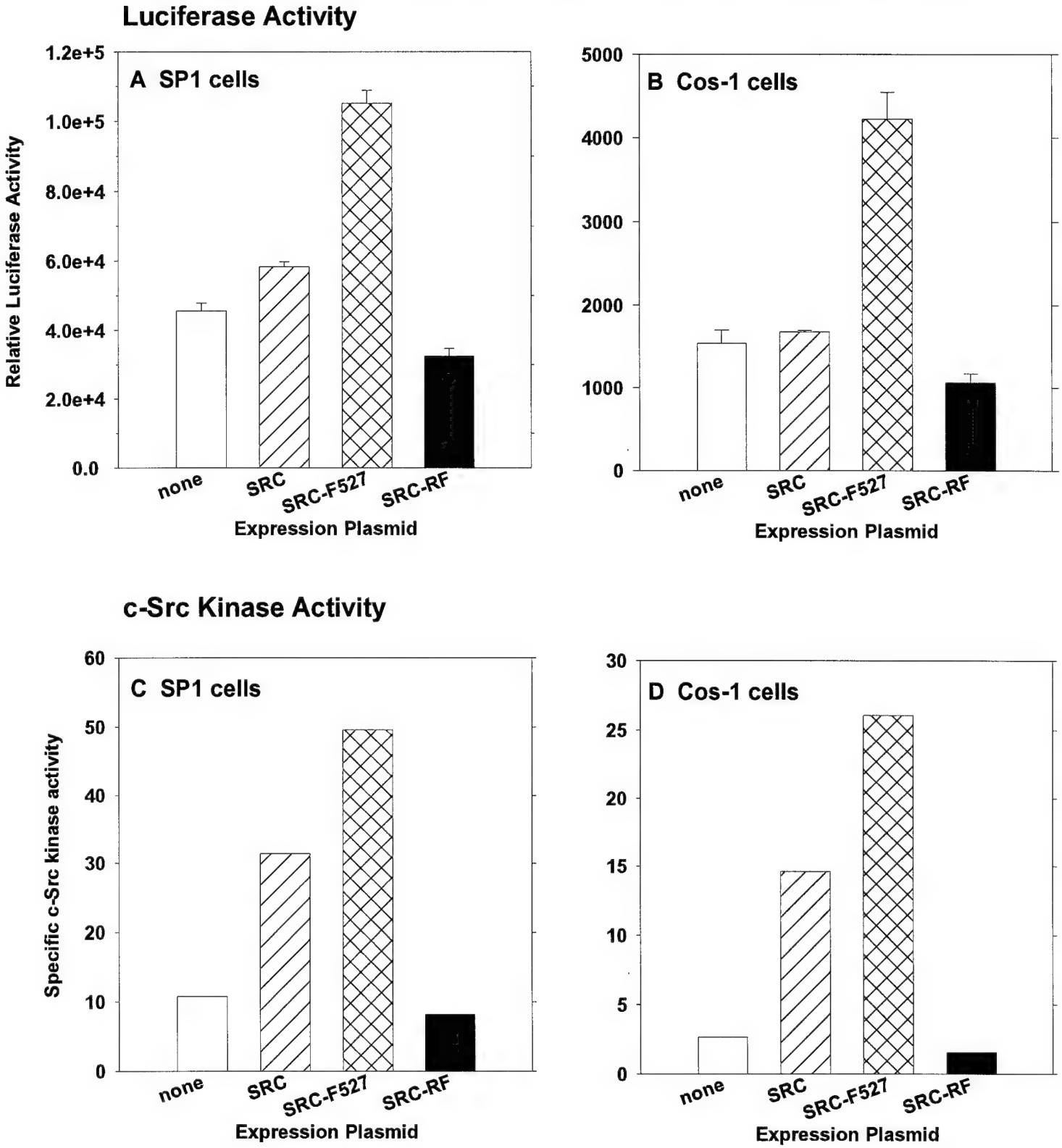
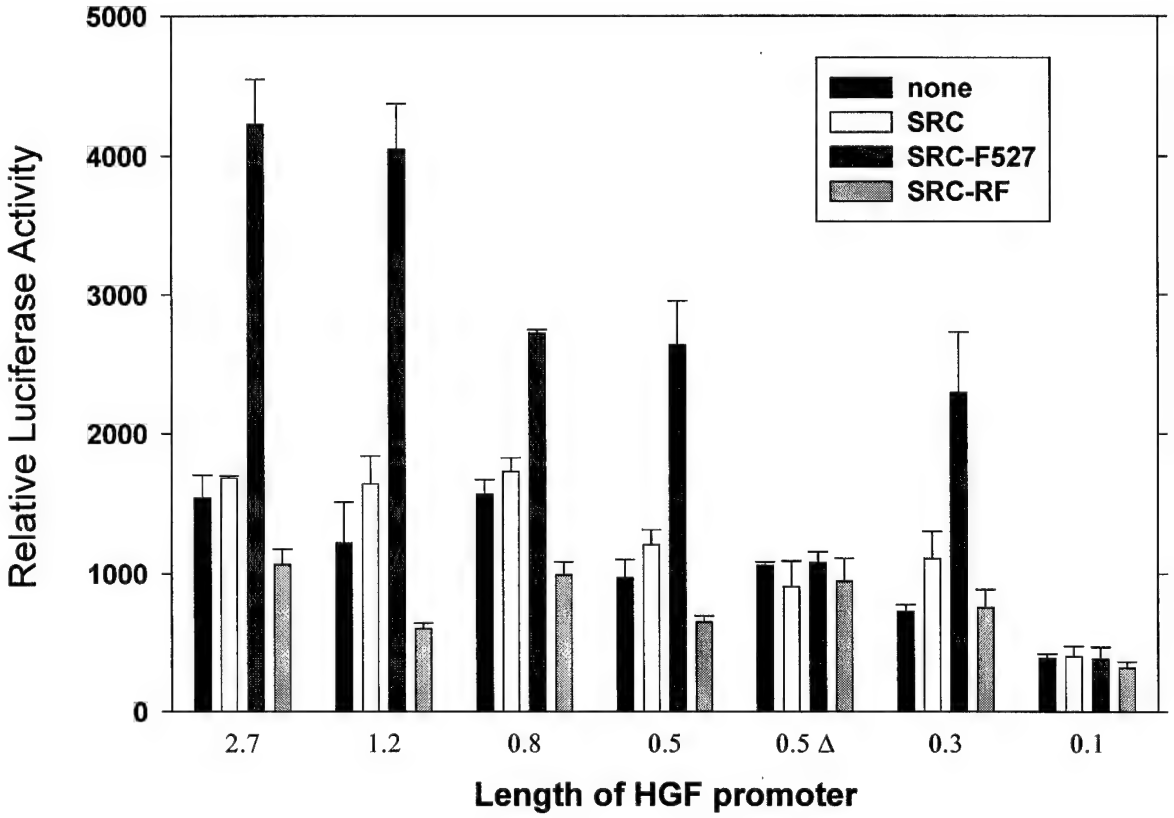


Figure 10

A **Mapping of c-Src Kinase Responsive Regions in the *HGF* Promoter**



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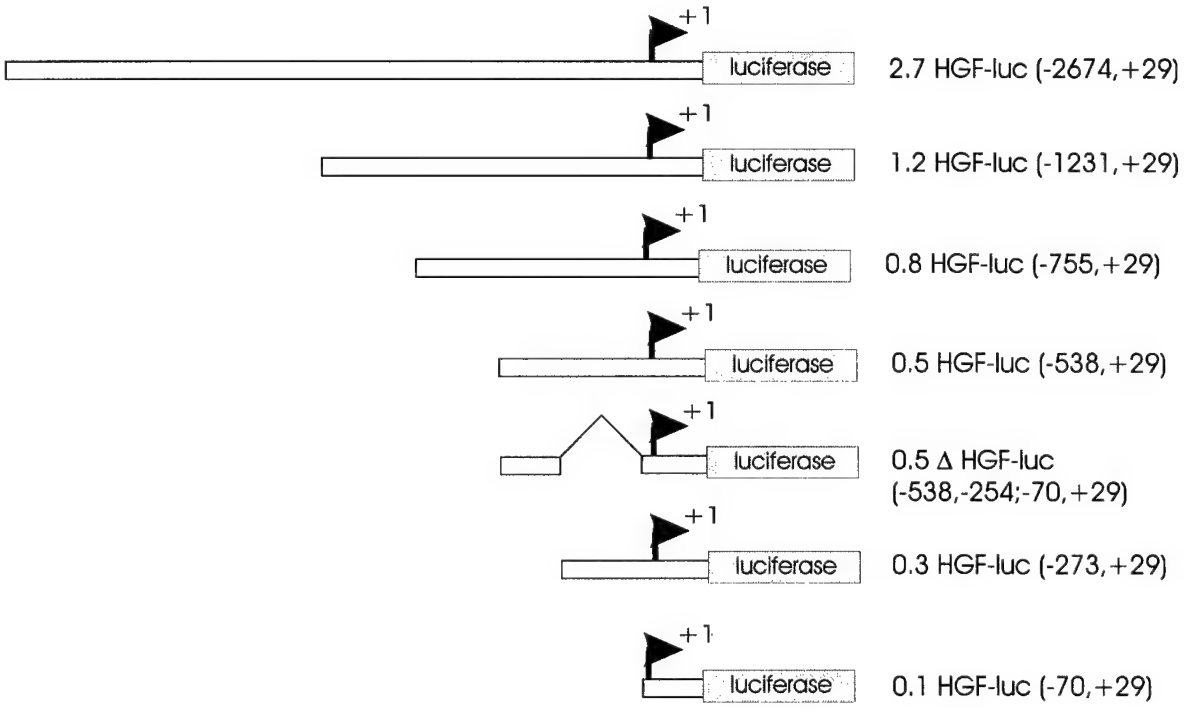


Figure 11

Proposed Organization of *HGF* Promoter Region

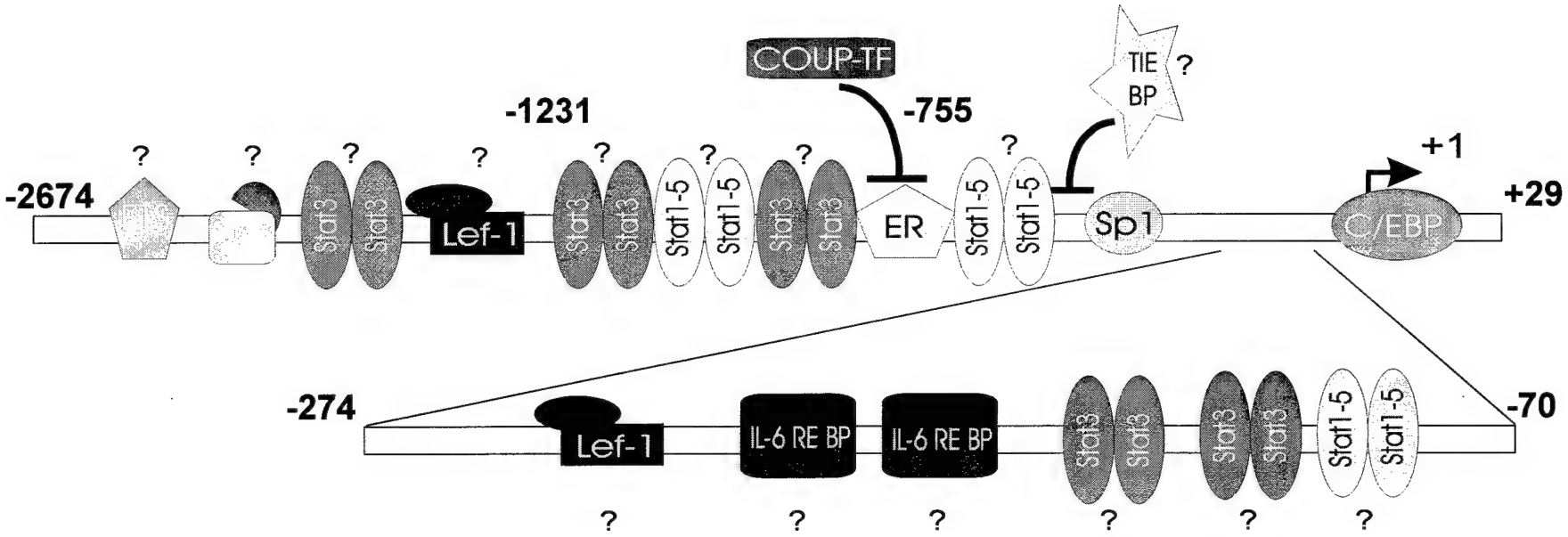
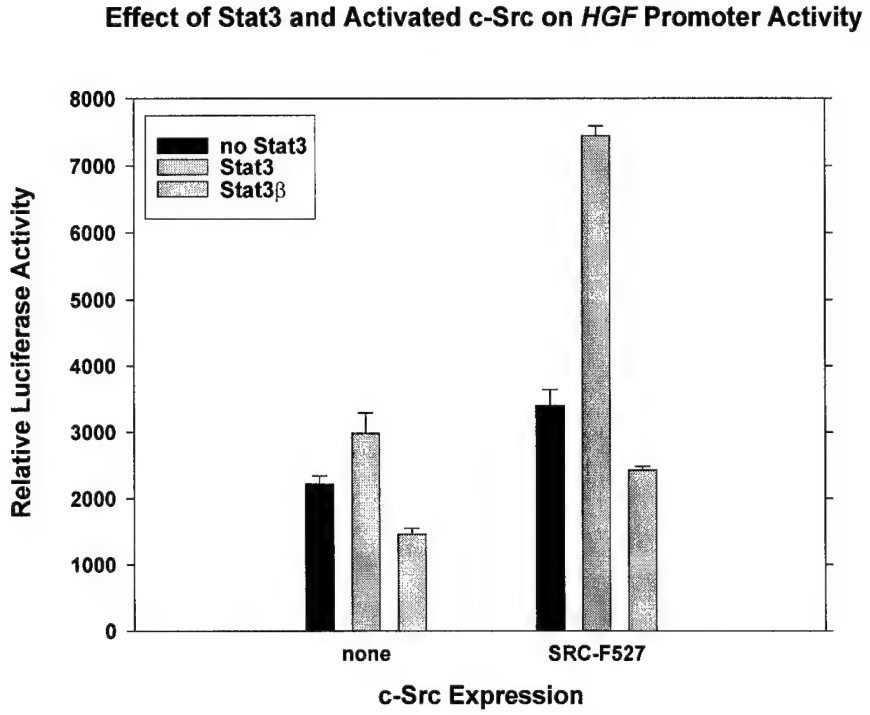
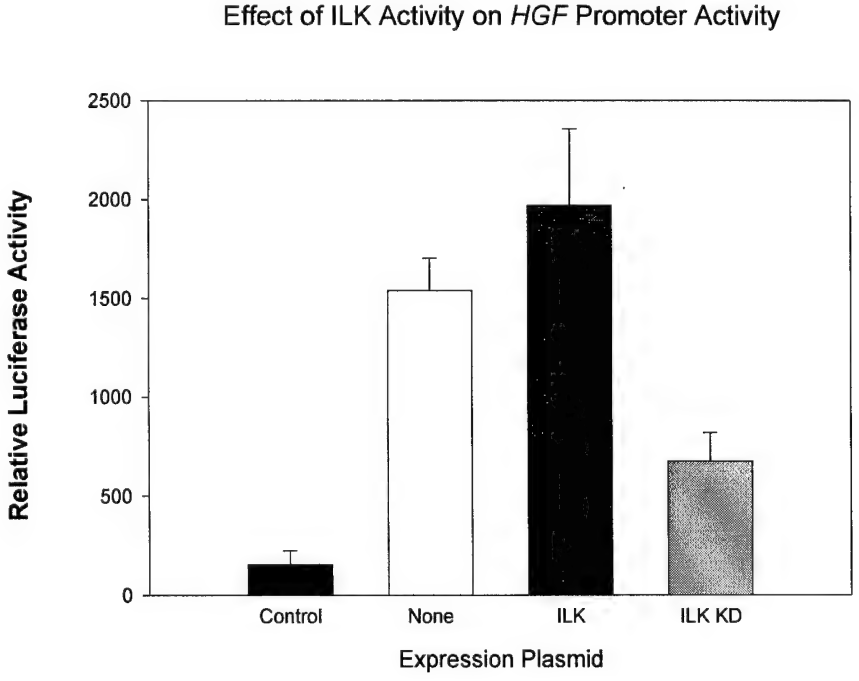


Figure 12

A


Effect of Stat3 and Activated c-Src on *HGF* Promoter Activity
no Stat3
Stat3
Stat3β
Relative Luciferase Activity
8000
7000
6000
5000
4000
3000
2000
1000
0
none
SRC-F527
c-Src Expression

B


Effect of ILK Activity on *HGF* Promoter Activity
Relative Luciferase Activity
2500
2000
1500
1000
500
0
Control
None
ILK
ILK KD
Expression Plasmid

Summary of key research accomplishment

- preliminary studies of the effect of EGF and TGFβ-1 on HGF mRNA expression using RT-PCR
- characterization of anti-HGF antibodies
- characterization of HGF expression in breast and lung carcinoma cell lines
- identification of novel HGF isoforms in one breast carcinoma cell line
- studies of the requirement of c-Src tyrosine kinase activity for HGF-induced motility and anchorage-independent growth of breast carcinoma cells
- identification of c-Src kinase responsive elements in the *HGF* promoter and the possible involvement of Stat3 in regulation of HGF expression

List of Publications and Abstracts Presented

Publication

N. Rahimi, **W. Hung**, R. Saulnier, E. Tremblay, and B. Elliott. c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. J. Biol. Chem., *273*: 33714-33721, 1998

Abstract Presented

1. J. Gui, J. Klassen, J. Ho, **W. Hung**, B. Campling, A. Boag, E. Sterns and B. E. Elliott. Identification of paracrine and possible autocrine hepatocyte growth factor loops in breast and non-small lung carcinomas. AACR Annual Meeting, New Orleans, Louisiana, 1998.

2. N. Rahimi, **W. Hung**, E. Tremblay, R. Saulnier, and B. Elliott. c-Src kinase activity is required for HGF-induced motility and anchorage-independent growth of mammary carcinoma cells. International Agency for Research on Cancer (IARC/WHO) Symposium on: Cell adhesion and communication in growth control and cancer. Lyon, France, 1999.

3. **W. Hung** and B. Elliott. c-Src tyrosine kinase activity is required for expression of hepatocyte growth factor in breast carcinoma cells. CBCRI Meeting "Breast Cancer Research: Reasons for Hope", Toronto, Ontario, Canada, 1999.

4. H. Qiao, **W. Hung**, N. Rahimi, L. Raptis, J. Rossiter, R. Saulnier, E. Tremblay, and B. Elliott. Co-operative interaction between hepatocyte growth factor and fibronectin in anchorage-independent survival and growth of breast carcinoma cells. CBCRI Meeting "Breast Cancer Research: Reasons for Hope", Toronto, Ontario, Canada, 1999.

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c-Src Kinase Activity Is Required for Hepatocyte Growth Factor-induced Motility and Anchorage-independent Growth of Mammary Carcinoma Cells*

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Overexpression and amplification of hepatocyte growth factor (HGF) receptor (Met) have been detected in many types of human cancers, suggesting a critical role for Met in growth and development of malignant cells. However, the molecular mechanism by which Met contributes to tumorigenesis is not well known. The tyrosine kinase c-Src has been implicated as a modulator of cell proliferation, spreading, and migration; these functions are also regulated by Met. To explore whether c-Src kinase is involved in HGF-induced cell growth, a mouse mammary carcinoma cell line (SP1) that co-expresses HGF and Met and a nonmalignant epithelial cell line (Mv1Lu) that expresses Met but not HGF were used. In this study, we have shown that c-Src kinase activity is constitutively elevated in SP1 cells and is induced in response to HGF in Mv1Lu cells. In addition, c-Src kinase associates with Met following stimulation with HGF. The enhanced activity of c-Src kinase also correlates with its ability to associate with Met. Expression of a dominant negative double mutant of c-Src (SRC-RF), lacking both kinase activity (K295R) and a regulatory tyrosine residue (Y527F), in SP1 cells significantly reduced c-Src kinase activity and strongly blocked HGF-induced motility and colony growth in soft agar. In contrast, expression of the dominant negative c-Src mutant had no effect on HGF-induced cell proliferation on plastic. Taken together, our data strongly suggest that HGF-induced association of c-Src with Met and c-Src activation play a critical role in HGF-induced cell motility and anchorage-independent growth of mammary carcinomas and further support the notion that the presence of paracrine and autocrine HGF loops contributes significantly to the transformed phenotype of carcinoma cells.

Evidence supports a role of hepatocyte growth factor (HGF)[1] and its receptor, the product of the *met* protooncogene, in both normal (1, 2) and malignant (3–5) epithelial cell development. In addition, a majority of human breast cancers show increased expression of HGF and Met (6–8), and this high level of HGF expression correlates with recurrence and poor patient survival (9). Met is also overexpressed in several other human cancers, including ovarian (10), melanoma (11), colon carcinomas (12), and osteosarcomas (13). Collectively, these observations suggest that activation of Met by overexpression, gene amplification, or establishment of an HGF autocrine loop may contribute to growth and development of mammary carcinomas. Previous studies demonstrated that co-expression of HGF and Met (4, 14), as well as expression of a constitutively active Met (Tpr-Met) in NIH-3T3 fibroblasts (15, 16) directly leads to cell transformation and tumorigenicity. However, the molecular mechanism by which HGF binding to its receptor elicits cell transformation is not fully understood.

A number of cytoplasmic signaling proteins, such as phosphatidylinositol (PI) 3-kinase, Grb2, Shc, Ras, and c-Src, have been shown to be involved in Met-dependent signal transduction pathways (17, 18). It is important to establish which of these signaling proteins regulate Met-dependent steps in tumor progression, because different signaling proteins may regulate various HGF-induced cellular functions, including mitogenic, motogenic, and morphogenic signals in target cells (18–22). The HGF-mediated signaling pathway is further complicated by the observation that the majority of SH2-containing cytoplasmic effectors bind to a single multifunctional docking site on the cytoplasmic domain of Met, whereas a second site is required for Grb2 binding (17, 18). Recent findings using a mutational approach demonstrated that different HGF-induced effects are regulated by these separate Met binding sites for cytoplasmic transducers (23–25) and that complementation in *trans* between these two binding sites is required for the invasive-metastatic phenotype (25). However, to study the role of specific SH2-containing cytoplasmic effectors in HGF receptor function, approaches to target individual cytoplasmic effectors are required. Recently, we (26) and others (27) have demonstrated that PI 3-kinase activity is required for HGF-induced mitogenic (26) and motogenic functions (27). These findings strongly argue that PI 3-kinase may play an important role in HGF-mediated growth of mammary carcinomas.

The tyrosine kinase c-Src is activated in response to HGF (17, 18) and other growth factors such as platelet-derived growth factor (PDGF) (28–30), fibroblast growth factor (31),

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[1] The abbreviations used are: HGF, hepatocyte growth factor; PI, phosphatidylinositol; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; PDGF, platelet-derived growth factor.

and epidermal growth factor (32). c-Src kinase activity is known to modulate cell proliferation (33, 34), spreading (35, 36), and migration (36–38) in many cell types; these functions are also regulated by HGF (19–23). c-Src kinase activity is increased 4-fold in human breast cancer (39, 40) and is also elevated in Neu-induced mouse mammary carcinomas in transgenic mice (41, 42). Activation of c-Src tyrosine kinase in transgenic mice induces mammary epithelial hyperplasias and is required, but is not sufficient, for induction of mammary tumors in polyoma virus middle T-transgenic mice (42, 43). Altogether, these observations support the notion that increased c-Src kinase activity in mammary carcinomas plays an important role in mammary tumor growth and development. However, the role of c-Src kinase in HGF-induced functions in mammary carcinoma cells is not clearly known.

To analyze whether c-Src kinase is involved in HGF-induced mammary carcinoma cell growth, we used a mouse mammary carcinoma cell line, SP1, which expresses HGF and tyrosine-phosphorylated Met, thereby generating an autocrine HGF loop in these cells (44). Our current results demonstrate that c-Src kinase activity is elevated in SP1 cells, compared with nonmalignant Mv1Lu epithelial cells. The increased activity of c-Src kinase correlates with its ability to associate with tyrosine-phosphorylated Met. We therefore examined the effect of expressing a dominant negative mutant form of c-Src on c-Src kinase activity and HGF-induced cell motility and anchorage-independent growth of SP1 carcinoma cells. Taken together, our findings show that c-Src kinase activation plays a significant role in HGF-induced cell motility and anchorage-independent growth, characteristics of the transformed phenotype.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit anti-sheep IgG conjugated to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (Westgrove, PN). Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KT). Rabbit anti-c-Src IgG, anti-Met (mouse) IgG, and anti-PLC-γ1 IgG were obtained from Santa Cruz Biotechnology (San Diego, CA).

Tissue Culture and Cell Lines—Mv1Lu cells are members of a mink lung epithelial cell line obtained from ATCC (Rockville, MA). Maintenance medium for Mv1Lu cells was Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% FBS. The SP1 tumor cell line is derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma and expresses HGF and Met. The characteristics of the SP1 cell line have been described elsewhere (45, 46). Maintenance medium for SP1 cells was RPMI 1640 (Life Technologies, Inc.) supplemented with 7% FBS (Life Technologies, Inc.).

Cell Transfection—cDNAs encoding wild type *c-src* (SRC) and a dominant negative double mutant of *c-src* (SRC-RF) with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F) ligated into the pRc/CMV plasmid (Invitrogen, San Diego, CA) carrying the neomycin resistance marker were obtained from Dr. J. Brugge (47). SP1 cells expressing the mutant c-Src and wild type c-Src were established using the stable transfection LipofectAMINE (Life Technologies, Inc.) method (48). Briefly, SP1 cells were grown to 80% confluence. The DNA (1 μg) was mixed with LipofectAMINE reagent (9 μl) in 200 μl of serum-free medium and was incubated for 15 min at room temperature. Before transfection, cells were washed once with 2 ml of serum-free medium. For each transfection, the mixed DNA and LipofectAMINE were combined with 0.8 ml of serum-free RPMI 1640 medium, and the cells were incubated with this transfection mixture. After 5 h of incubation, an equal volume of RPMI/14% FBS was added to the transfection medium, and incubation proceeded for an additional 24 h. For most experiments, pooled transfected cells selected with G418 (450 μg/ml) were used. In one experiment, SP1 cells were transfected with SRC-RF or SRC, and clones were isolated and tested for Src kinase activity and colony forming efficiency.

Cell Proliferation and Colony Growth Assay—Cell proliferation was carried out as described elsewhere (45). Briefly, SP1 carcinoma cells and Mv1Lu cells were plated at 10^4 cells/well in 24-well plates under the various conditions indicated. DNA synthesis was measured by adding 0.2 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Oakville, ON, Canada) at 24 h. After an additional 24 h, cells were harvested with trypsin/EDTA. Aliquots of cells were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (ICN, Costa Mesa, CA), and [^{3}H]thymidine incorporation was measured in a scintillation counter (Beckman, Mississauga, ON, Canada). Results are expressed as the mean cpm/well ± S.D. of triplicates.

Colony growth assays were performed as described previously (49). Briefly, a solution of 1.2% Bactoagar (Difco Lab) was mixed (1:1) with 2× RPMI 1640, supplemented with FBS at final concentrations of 7 or 1% alone or with HGF as indicated, and layered onto 60 × 15-mm tissue culture plates. SP1 cells (10^3/2.5 ml) were mixed in a 0.36% Bactoagar solution prepared in a similar way and layered (2.5 ml/plate) on top of the 0.6% Bactoagar layer. Plates were incubated at 37 °C in 5% CO_2 for 8–10 days. Colonies were fixed with methanol, stained with Giemsa, and counted manually. Results are expressed as mean number of colonies per dish ± S.D. of quadruplicates.

Cell Motility Assay—To measure cell motility, Transwell culture inserts (8-μm pore size) (Costar, Toronto, ON, Canada) were coated uniformly with gelatin (0.25% w/v, Sigma, Oakville, ON, Canada) on both sides for 2 min at room temperature (50). Membranes were washed twice with serum-free RPMI 1640 medium and inserted into a 24-well culture plate (Costar, Toronto, ON, Canada) with 1 ml of RPMI 1640 containing 0.5 mg/ml bovine serum albumin (Life Technologies, Inc.). Cells were grown to 50% confluence, serum-starved overnight, and harvested in 5 mM EDTA. Cells (2×10^4/100 μl) were plated in the insert and incubated for 6–8 h at 37 °C. Following the incubation, excess medium was removed, and cells were fixed in 1% paraformaldehyde (Sigma) for 15 min and stained with hematoxylin (Fisher, Oakville, ON, Canada). Cells on the upper side of the membrane were removed by wiping with cotton. Cells on the under side of the membrane were counted using an inverted microscope with phase contrast illumination. Cell motility is expressed as the number of migrating cells per well. In a parallel study, a wounding assay was performed, as described previously (36). Briefly, monolayers of each cell type were "wounded" by scraping with an Eppendorf yellow tip, washed, and incubated alone or with HGF for varying times. Migration was assessed visually by the ability of cells to close the wounded area.

Immunoprecipitation and Western Blotting—Cells were grown to confluence and serum-starved for 24 h. Cells were rinsed with cold phosphate-buffered saline three times and lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4 °C. Protein concentration of supernatants was determined using a bicinchoninic acid protein assay (Pierce). Equal protein amounts from each cell lysate were incubated with the indicated antibodies at 4 °C for 2 h or overnight. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min with 3% skimmed milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and probed for 1 h with the indicated antibodies. The membrane was washed three times for 5 min each with TBST buffer, incubated with horseradish peroxidase-labeled secondary anti-rabbit or anti-mouse antibodies for 15 min, and washed three times with TBST for 10 min each time. Immune complexes were detected using ECL (Amersham).

In Vitro c-Src Kinase Assay—In most experiments, an *in vitro* c-Src kinase assay using enolase as a substrate was performed as described previously (51). Briefly, lysates from SP1 and Mv1Lu cells were prepared, and equal protein amounts from each cell lysate were immunoprecipitated with anti-c-Src IgG (Santa Cruz Biotechnology) as described above. The amount of anti-c-Src IgG was pre-determined to be in excess over c-Src protein, indicating that the majority of c-Src protein in cell lysates is immunoprecipitated (data not shown). One-half of each immunoprecipitate was subjected to SDS-PAGE under nonreducing conditions and Western blot analysis to confirm the amount of c-Src protein present. The other half of each immunoprecipitate was assayed for c-Src kinase activity, by incubating with 10 μl of reaction buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4), 1 μl of freshly prepared acid-denatured enolase (Sigma) (5 μg of enolase + 1 μl of 50 mM HCl incubated at 30 °C for 10 min then neutralized with 1 μl of 1 M PIPES, pH 7.0), and 10 μCi of [γ-^{32}P]ATP. After 10 min of incubation at 30 °C, reactions were terminated by the addition of 2× SDS sample buffer, and samples were subjected to 8% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature and drying for 2 h at

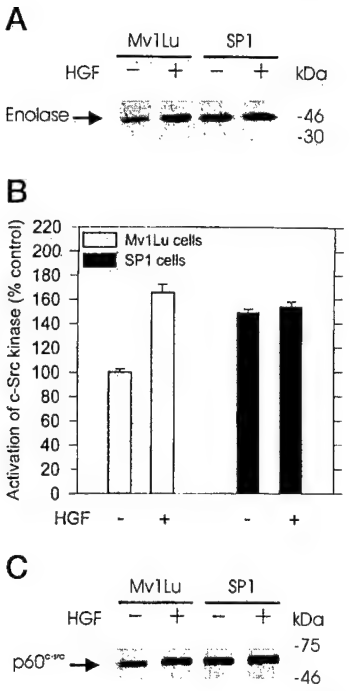

FIG. 1. **c-Src kinase activity is elevated in SP1 carcinoma cells compared with Mv1Lu epithelial cells.** Cell lysates were prepared from serum-starved Mv1Lu and SP1 cells treated without (−) or with (+) HGF (40 ng/ml) for 10 min and were immunoprecipitated with anti-c-Src IgG. Immunoprecipitates were subjected to an *in vitro* kinase assay using enolase as a substrate, and kinase activity was measured as described under "Experimental Procedures." *A*, autoradiogram showing ^{32}P-labeled enolase. *B*, quantitation of autoradiogram using PhosphorImager. Results are expressed as the percentage of cpm in untreated Mv1Lu cells (100%), normalized to the amount of c-Src protein in *C*. The means ± range of two experiments are shown. Similar results were obtained using the c-Src kinase family-specific cdc2 peptide as substrate (data not shown). *C*, Western blot analysis of immmunoprecipitates in *A*, probed with anti-c-Src IgG.

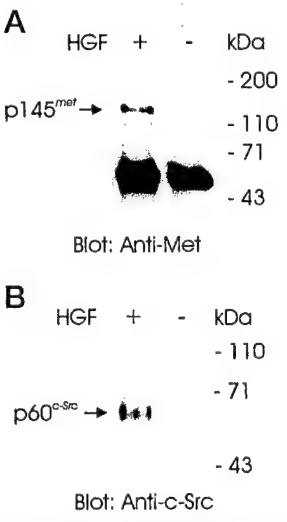

FIG. 2. **c-Src kinase binds to tyrosine-phosphorylated Met.** Cell lysates derived from serum-starved Mv1Lu cells treated without (−) or with (+) HGF (40 ng/ml) for 15 min were immunoprecipitated with anti-c-Src IgG (*A*) or anti-Met IgG (*B*). The immune complexes were separated by 8% SDS-PAGE and immunoblotted with anti-Met IgG (*A*) or anti-c-Src IgG (*B*). Protein molecular mass standards are shown on the *right*. This experiment was done twice with similar results.

80 °C under a vacuum. Autoradiograms were produced and quantitated using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In some experiments (see Fig. 3), c-Src kinase activity was assayed according to Cheng *et al.* (52) using the c-Src tyrosine kinase family-

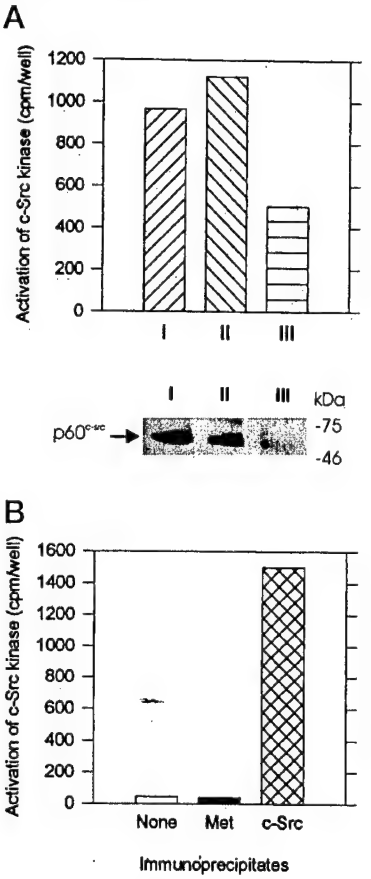

FIG. 3. **Detection of c-Src kinase activity in Met immunoprecipitates.** *A*, equal amounts of cell lysates derived from serum-starved SP1 cells were immunoprecipitated with anti-c-Src antibody (*bar I*) or anti-Met antibody (*bar II*). The supernatant from immunoprecipitates of anti-Met antibody was subsequently immunoprecipitated with anti-c-Src antibody (*bar III*). *In vitro* c-Src kinase activity was determined as described under "Experimental Procedures" using the c-Src kinase family-specific cdc2 peptide substrate. The amount of radiolabeled cdc2 substrate was determined and plotted as c-Src kinase activity (cpm/well) (*top panel*). Half of each immunoprecipitate in the *top panel* was subjected to SDS-PAGE, and p60$^{c\text{-}src}$ protein in each sample was identified by immunoblotting with anti-c-Src antibody (*bottom panel*). *B*, equal amounts of cell lysates derived from serum-starved SP1 cells were immunoprecipitated with anti-Met antibody or anti-c-Src antibody under more stringent conditions with RIPA buffer to prevent co-precipitation of other proteins (see "Experimental Procedures"). The immunoprecipitates were used in an *in vitro* c-Src kinase assay with the c-Src kinase family-specific cdc2 substrate. As a control, a reaction containing no protein (*None*) was carried out concurrently. Results are plotted as c-Src kinase activity (cpm/well) as in *A*. Anti-Met immunoprecipitates under these more stringent conditions showed no significant phosphorylation of the cdc2 substrate.

specific cdc2 peptide substrate. Anti-c-Src or anti-Met immunoprecipitates prepared as above were incubated with 40 μl of a reaction buffer (100 mM Tris-HCl, pH 7.0, 0.4 mM EGTA, 0.4 mM Na_3VO_4, 40 mM $Mg(OAc)_2$), 5 μl of cdc2 peptide (Life Technologies, Inc., 250 μM/assay), 5 μl of cold ATP (25 μM), and 2.5 μCi of [γ-^{32}P]ATP. A control consisting of immunoprecipitation with anti-Met IgG under more stringent conditions with RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) where c-Src would not be co-precipitated was also carried out. After 15 min of incubation at 37 °C, reactions were terminated by the addition of 20 μl of 40% trichloroacetic acid and incubated for an additional 5 min. Aliquots subsequently were blotted on to p81 paper (Whatman, Fisher, Ottawa, ON, Canada). The p81 paper was washed three times (5 min/wash) with 0.75% phosphoric acid and once with acetone at room temperature, and the radiolabeled c-Src kinase substrate was counted in a liquid scintillation counter.

In Vitro Met Kinase Assay—Cell lysates from SP1 and Mv1Lu cells were prepared, and equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG as described above. Immunoprecipitates were washed twice with cold lysis buffer and once with cold kinase

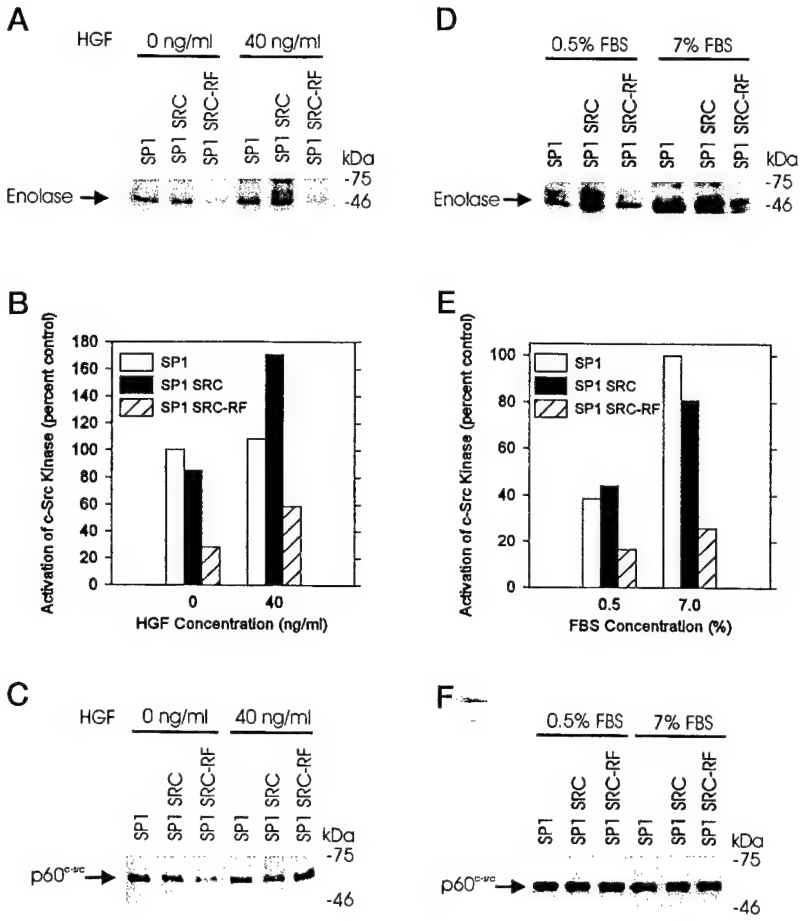

FIG. 4. **Effect of transfected dominant negative SRC-RF on Src kinase activity in SP1 cells.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were plated at 70% confluence and prestarved overnight. Cells in each group were then cultured alone, with HGF (40 ng/ml), or with 0.5 or 7% FBS, and an *in vitro* c-Src kinase assay using enolase as a substrate was performed as described under "Experimental Procedures." *A* and *D*, autoradiograms showing ^{32}P-labeled enolase. *B*, and *E*, quantitation of autoradiogram using densitometry. Results are normalized to amount of c-Src protein in *C* and *F*. *C*, and *F*, Western blot analysis of immmunoprecipitates in *A* and *D*, probed with anti-c-Src IgG. This result is representative of five experiments.

buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4). *In vitro* Met kinase activity was determined by incubating immunoprecipitates with 20 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP at 30 °C for 10 min. The reaction was stopped by addition of 2× SDS sample buffer containing 5% β-mercaptoethanol. Samples were boiled for 3 min and subjected to 7% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing and drying as described above. Autoradiograms were produced and quantitated using a Storm PhosphorImager (Molecular Dynamics).

RESULTS

Detection of Elevated c-Src Tyrosine Kinase Activity in SP1 Carcinoma Cells—SP1 carcinoma cells express HGF and tyrosine-phosphorylated Met, consistent with an HGF autocrine loop in these cells (44). To test the possibility that activation of c-Src kinase may be involved in Met-induced signaling pathways, we measured the kinase activity of c-Src in SP1 carcinoma cells and an HGF-sensitive epithelial cell line, Mv1Lu. c-Src kinase activity was measured by the capacity of c-Src immunoprecipitates from these cells to tyrosine phosphorylate the substrate, enolase. c-Src immunoprecipitates from serum-starved SP1 cells showed a pronounced elevated kinase activity, which increased only slightly following treatment with exogenous HGF (Fig. 1). In contrast, c-Src kinase activity in Mv1Lu cells was highly dependent on stimulation of cells with exogenous HGF (Fig. 1). The levels of c-Src kinase activity observed correlated with the constitutive tyrosine phosphorylation of Met (44) and *in vitro* Met kinase activity (data not shown) in SP1 cells, and the HGF-induced tyrosine phosphorylation of Met in Mv1Lu cells (Ref. 26 and data not shown).

Association of c-Src Kinase Protein and Activity with Activated Met—It is conceivable that the high level of c-Src kinase activity in SP1 cells, could have resulted from interaction of c-Src with activated Met due to an autocrine HGF loop in these cells (44). To test for interaction of c-Src kinase family proteins with activated *versus* nonactivated Met, we first examined the association of c-Src with Met in Mv1Lu cells that express Met but not HGF. Serum-starved Mv1Lu cells were incubated alone or with HGF, and cell lysates were immunoprecipitated with anti-Met IgG or anti-c-Src IgG. Protein precipitates were electrophoresed and subjected to Western blotting with anti-c-Src IgG or anti-Met IgG, respectively. As shown in Fig. 2 (*A* and *B*), an increased amount of c-Src protein was recovered from anti-Met immunoprecipitates and *vice versa* in cell lysates from HGF-treated Mv1Lu cells compared with untreated Mv1Lu cells. We also showed that association of c-Src kinase with Met occurred via the SH2 domain of c-Src and correlated with tyrosine phosphorylation of Met (data not shown). It should be noted that a trace amount of c-Src protein was detected in lysates of unstimulated cells immunoprecipitated with anti-Met IgG and blotted with anti-c-Src IgG, possibly due to incomplete starvation of these cells before HGF stimulation (Fig. 2*B*). Thus, stimulation with HGF causes increased association of c-Src protein with Met.

To determine whether elevated activity of c-Src kinase in SP1 cells correlates with its ability to associate with Met, serum-starved SP1 cells were immunoprecipitated with anti-Met IgG or anti-c-Src IgG, and immunoprecipitates were tested for the ability to tyrosine phosphorylate the c-Src kinase family-specific cdc2 peptide substrate (52). As shown in Fig. 3*A* (*bars I* and *II*), similar amounts of c-Src kinase protein and activity were recovered from immunoprecipitates of both anti-Met and anti-c-Src antibodies. In contrast, immunoprecipitates from anti-Met IgG under more stringent conditions with RIPA buffer where c-Src is not co-precipitated resulted in no signifi-

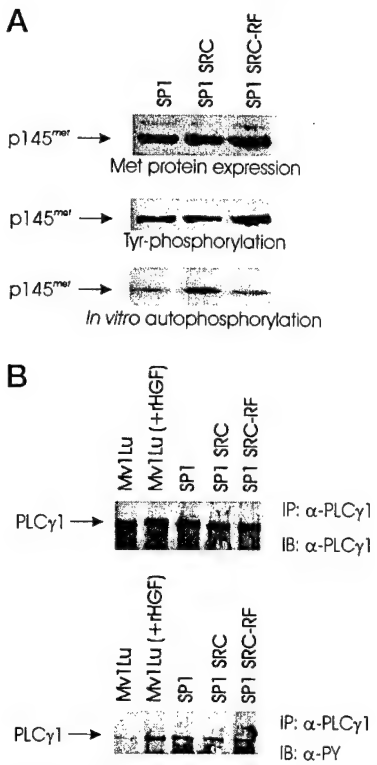

FIG. 5. **Expression of dominant negative mutant SRC-RF does not alter Met protein levels or activity and downstream signaling.** *A*, SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were prestarved overnight and lysed as described in the legend to Fig. 1. Equal amounts of protein from each lysate were concentrated on Microcon 10 filters (Amicon Inc., Beverly, MA) and analyzed by Western blotting with anti-Met IgG (*top panel*). The blot was stripped and reprobed with anti-phosphotyrosine antibody (*middle panel*). Cell lysates were also immunoprecipitated with anti-Met IgG, and immunoprecipitates were subjected to an *in vitro* Met kinase assay as described under "Experimental Procedures." The autoradiogram depicting ^{32}P-labeling of Met is shown (*bottom panel*). Relative band intensities and amount of ^{32}P labeling was determined using a Storm PhosphorImager. The relative amount of Met tyrosine phosphorylation (1.0, 1.0, or 1.0) or of *in vitro* Met autophosphorylation (1.0, 1.1, or 1.0) was not significantly different among the three cell lines. *B*, serum-starved SP1 cells transfected with SRC-RF or SRC and untreated SP1 cells were lysed as described in the legend to Fig. 1. Prestarved Mv1Lu cells untreated or treated with HGF (40 ng/ml) for 10 min were used as negative and positive controls, respectively. Equal amounts of protein from each lysate were immunoprecipitated with anti-PCL-γ1 IgG. Immunoprecipitates were subjected to 7% SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-PCL-γ1 IgG (*top panel*) before being stripped and reprobed with anti-phosphotyrosine antibody (*bottom panel*). This experiment was done twice with similar results. *IP*, immunoprecipitation; *IB*, immunoblot.

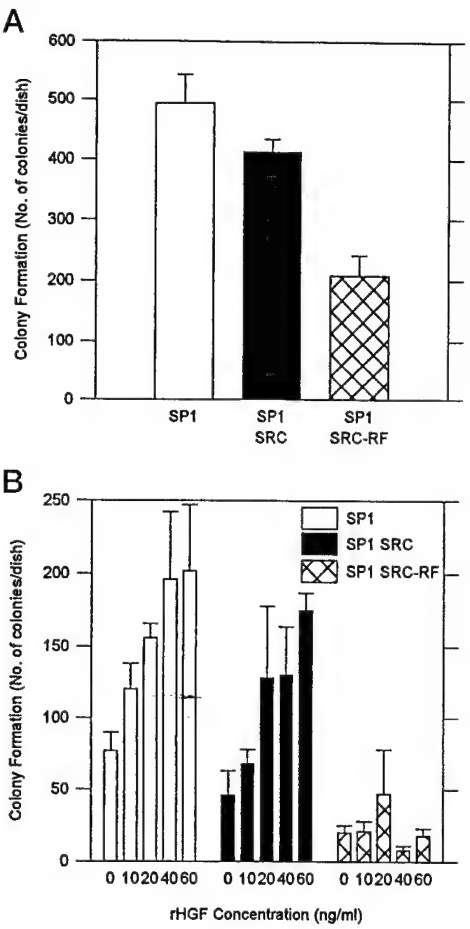

FIG. 6. **Effect of transfected dominant negative SRC-RF on growth of SP1 cells in agar.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were cultured (10^3 cells/dish) in 60-mm tissue culture plates in soft agar (0.36%) with RPMI 1640 medium supplemented with 7% FBS (*A*) or 1% FBS plus HGF at the concentrations indicated (*B*) as described previously (49). After 8 days, colonies were stained with Giemsa and counted visually. Results are expressed as the mean colony numbers ± S.D. of quadruplicate cultures. This experiment was done three times with similar results.

cant phosphorylation of cdc2 peptide (Fig. 3*B*), confirming the specificity of the cdc2 peptide as a substrate for c-Src (52). Thus a significant portion of c-Src kinase activity is associated with activated Met in SP1 cells. To further evaluate the contribution of c-Src association with Met, the supernatant from the immunoprecipitate of anti-Met IgG was immunoprecipitated for a second time with anti-c-Src IgG and subjected to the *in vitro* c-Src kinase assay. As shown in Fig. 3*A* (*bar III*), some c-Src kinase activity was detected in the Met-depleted SP1 cell lysate; however, it was with much lower activity, corresponding to the reduced amount of c-Src protein present. Immunoprecipitation of SP1 cell lysates with higher concentrations of anti-Met IgG and subsequently with anti-c-Src IgG showed a similar result (data not shown). These results demonstrate that the majority of c-Src kinase activity correlates with its ability to associate with Met in SP1 cells.

c-Src Kinase Activity Is Required for Colony Growth in Agar, but Not Cell Proliferation on Plastic—SP1 cells exhibit paracrine stimulation by HGF of colony growth in agar and proliferation on plastic (45, 49). To determine whether c-Src kinase activity is required for HGF-induced proliferation or colony growth in agar, an expression vector (SRC-RF) containing cDNA encoding a dominant negative double mutant of *c-src* with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F) (47) was stably transfected into SP1 cells. A control consisted of cells transfected with the same vector expressing wild type *c-src* (SRC). Uncloned (pooled) transfected cells were selected in G418-containing medium and assessed for c-Src kinase activity and HGF-induced functions. For *in vitro* c-Src kinase assays, immunoprecipitation with anti-c-Src IgG was carried out at antibody excess, indicating that the majority of wild type c-Src protein was present in immunoprecipitates. The results showed that c-Src kinase activity was strongly reduced in SRC-RF transfected SP1 cells compared with SRC transfected or untransfected cells incubated alone, or following stimulation with 40 ng/ml HGF or 7% FBS (Fig. 4). However, tyrosine phosphorylation of Met or an unrelated signaling molecule PLC-γ1 and *in vitro* autophosphorylation of Met remained unaffected in SRC-RF- or SRC-transfected SP1 cells, compared with untransfected cells (Fig. 5). These results demonstrate

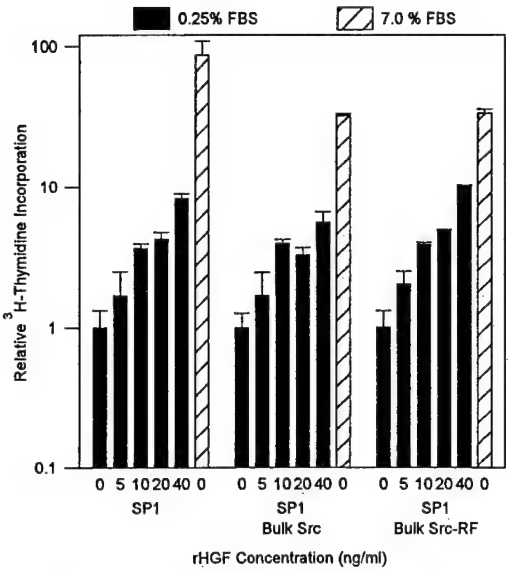

FIG. 7. **Effect of transfected dominant negative mutant SRC-RF on HGF-induced proliferation of SP1 cells on plastic.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were prestarved overnight, and each cell line was plated at 10^4 cells/well in 24-well plates in 0.25% FBS without or with HGF at the concentrations indicated. Controls consisted of cultures with 7% FBS. DNA synthesis was measured as described under "Experimental Procedures." Results are expressed as relative mean [^{3}H]thymidine incorporation compared with control (no HGF) (mean cpm/well ± S.D. of triplicates). This result is representative of four experiments.

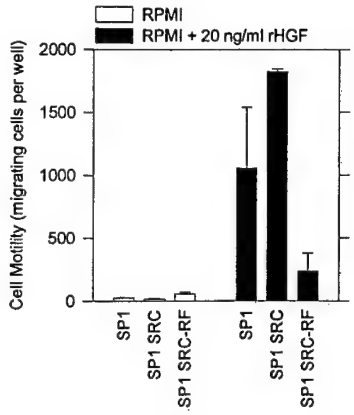

FIG. 8. **Effect of transfected dominant negative SRC-RF on HGF-induced cell motility.** SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were serum-starved overnight, and each cell line (2×10^4 cells) was plated into Transwell inserts (8-μm pore size) in 24-well plates in 0.5 mg/ml bovine serum albumin in RPMI without (*open bars*) or with (*closed bars*) HGF (20 ng/ml) as described under "Experimental Procedures." After 6–8 h of incubation at 37 °C, cells were fixed in 1% paraformaldehyde and stained with hematoxylin. Cells on the upper side of the membrane were removed by wiping with cotton. Cells on the underside were counted using an inverted microscope with phase contrast illumination. The results are expressed as the relative number of migrating cells/well (means ± range of two wells/point). This experiment was done twice with similar results. Similar results were obtained using a wound healing assay (data not shown).

specificity of the inhibitory effect of SRC-RF on c-Src kinase activity.

Expression of the dominant negative SRC-RF mutant in SP1 cells significantly inhibited FBS- and HGF-induced colony formation in soft agar, compared with SRC-transfected or untransfected SP1 cells (Fig. 6). Similarly, a subclone of SP1 cells expressing SRC-RF showed a marked reduction in colony formation, compared with a wild type SRC-transfected subclone or untransfected SP1 cells (data not shown). In contrast, SRC-RF-transfected SP1 cells showed no difference in HGF-induced or serum-induced proliferation on plastic, compared with SRC-transfected or untransfected SP1 cells (Fig. 7). Thus reduction of c-Src kinase activity in SRC-RF-transfected cells abrogated HGF- or serum-induced colony growth in soft agar but had no effect on cell proliferation on plastic.

c-Src Kinase Activity Is Required for HGF-induced Cell Motility—Because c-Src kinase activity has been shown to modulate cell motility in several cell types (36–38), we examined the role of c-Src kinase in HGF-induced cell motility in SP1 cells. Our results showed that HGF strongly stimulated motility of SP1 cells through collagen-coated porous membranes in a paracrine manner. HGF-induced motility was significantly reduced in SP1 cells transfected with dominant negative mutant SRC-RF, compared with SRC-transfected or untransfected cells (Figs. 8 and 9). Similar results were obtained using a wounding assay (data not shown). These results are consistent with a role of c-Src kinase in HGF-induced cell motility.

DISCUSSION

We (6) and others (7, 8) have previously shown that HGF and Met mRNA are strongly co-expressed in invasive carcinomas in human breast cancer. These findings suggest that signals transduced by activated Met confer survival and growth advantage to carcinoma cells during progression to metastasis. This concept is further supported by the observation that cells transfected with an activated version of *met* (*tpr-met*) acquire invasive and metastatic properties (15, 16). Unlike most other receptor tyrosine kinases, Met shows one high affinity binding site for the majority of SH2-containing cytoplasmic effectors, suggesting that these proteins bind Met in a competitive manner (23–25). Therefore, to study the role of specific SH2-containing cytoplasmic effectors in HGF receptor function, approaches to target individual cytoplasmic effectors are required.

To analyze downstream effector molecules in HGF-induced tumorigenic properties of mammary carcinoma cells, we have studied a mouse mammary carcinoma, SP1, which co-expresses HGF and Met (44). However, depending on culture conditions, both paracrine and autocrine effects of HGF have been observed in SP1 cells (44, 45, 49). In monolayer cultures, autocrine phosphorylation of Met at tyrosine in SP1 cells without addition of exogenous HGF was observed (44). In contrast, tyrosine phosphorylation of Met was reduced in suspended SP1 cells and can be restored by addition of exogenous HGF.[2] These observations suggest that the base level of Met activation may be influenced by extracellular environmental conditions, such as cell adhesion to various substrata (53), cell density effects on HGF expression and secretion (54), or proteolytic processing of pro-HGF to the biologically active form (55). In the present report, paracrine stimulation with exogenous HGF was required for optimal cell proliferation, motility, and colony growth in agar under serum-starved conditions. Previous studies showed that PI 3-kinase activity is elevated in SP1 cells and that its activity is required for HGF-induced proliferation in monolayer culture. Treatment of SP1 cells with wortmannin, a potent inhibitor of PI 3-kinase (56), or transfection of a dominant negative mutant of the p85 subunit of PI 3-kinase into these cells (26) inhibited HGF-induced cell proliferation in monolayer culture.

In the present report, we show that c-Src kinase activity is elevated in SP1 mammary carcinoma cells compared with non-malignant Mv1Lu epithelial cells and is associated with Met. The elevated level of c-Src kinase activity in SP1 cells and its association with Met strongly suggest that this signaling mol-

[2] R. Saulnier and H. Qiao, unpublished observation.

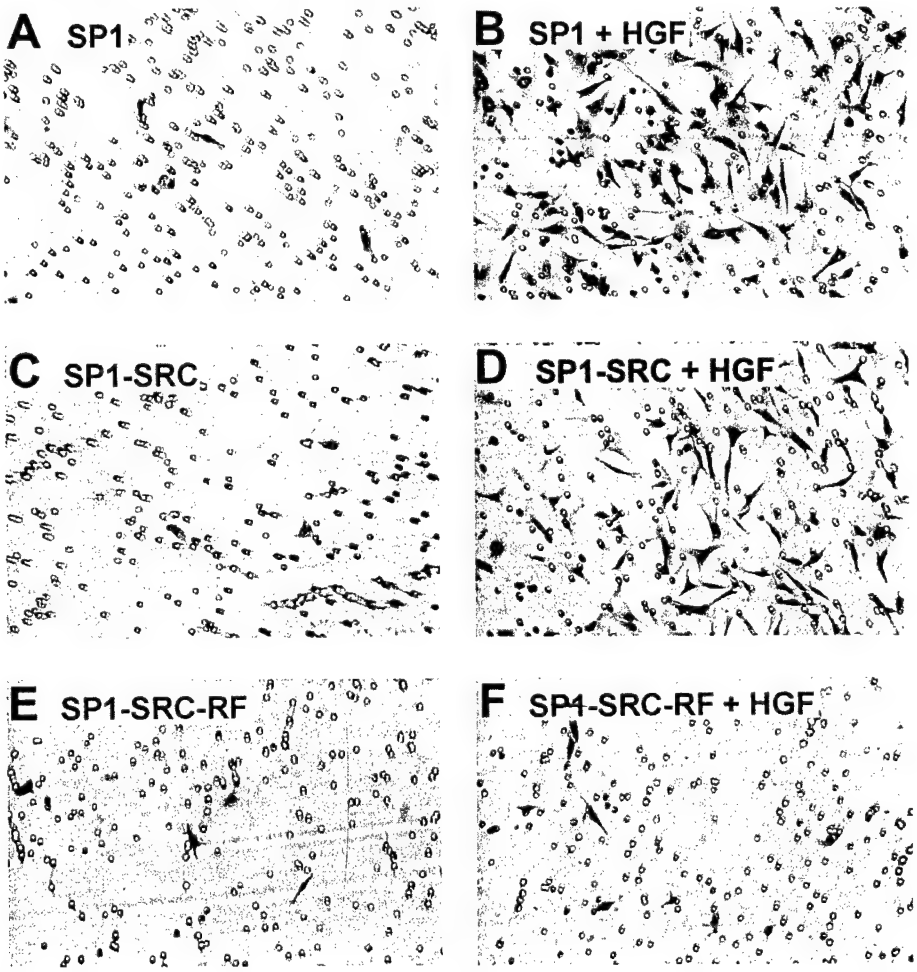

FIG. 9. **Photomicrographs of migrating SP1 cells transfected with SRC or SRC-RF or untransfected SP1 cells following HGF stimulation.** SP1 cells untransfected (*A* and *B*) and transfected with SRC (*C* and *D*) or SRC-RF (*E* and *F*) were serum-starved overnight and assessed for cell motility without (−) or with (+) HGF (20 ng/ml) as described in the legend to Fig. 8. After removing nonmigrating cells on the upper side of the membrane, membranes were mounted onto glass slides, and migrating cells were photographed using a Leitz microscope with phase contrast illumination. Photographs correspond to the groups shown in Fig. 8. Original magnification, 250×.

ecule may be involved in intracellular events triggered by HGF. This observation prompted us to test whether expression of a dominant negative mutant form of c-Src influences growth of SP1 cells. Expression of a dominant negative form of c-Src (SRC-RF) in SP1 cells showed no significant effect on HGF-induced cell proliferation on plastic but markedly inhibited HGF- or serum-induced colony growth in soft agar. Thus activation of c-Src kinase is essential for colony formation in agar by SP1 cells but appears not to be required for cell proliferation on plastic.

Other laboratories have reported variable effects of c-Src kinase on cell growth. In support of our observations, Demali and Kazlauskas (57) have shown that a mutant form of PDGF β-receptor that cannot bind or activate, c-Src, retains the ability to stimulate growth of fibroblasts on plastic or in agar in response to PDGF. In contrast, Courtneidge and co-workers (33, 58) have shown that microinjection of a kinase dead mutant c-Src or neutralizing antibodies that inhibit basal and stimulated c-Src kinase activity inhibited PDGF-dependent DNA synthesis in fibroblasts. Similarly, constitutive expression of c-Src mutants inhibited PDGF and epidermal growth factor-induced mitogenesis of mouse embryonal fibroblasts lacking c-Src (34). The apparent differences in the role of c-Src kinase in the above systems could be due to different levels of residual basal activity of c-Src kinase or the developmental and malignant status of the cells used. Our observation that anchorage-independent growth but not proliferation on plastic is inhibited in cells expressing dominant negative SRC-RF suggests that the reduced level of c-Src kinase activity in SRC-RF expressing SP1 cells is insufficient to support anchorage-independent growth, whereas proliferation on plastic remains unaffected. c-Src-independent signaling mechanisms may also promote HGF-induced proliferation of SP1 cells on plastic.

We have also shown that SP1 cells transfected with the dominant negative SRC-RF mutant showed reduced cell motility in response to HGF compared with SRC-transfected or untransfected SP1 cells. Thus c-Src kinase activity is required for HGF-induced cell motility in SP1 carcinoma cells, although complementary signaling molecules may also be involved. This observation reflects recent reports that c-Src kinase activity is required for epithelial cell scattering (38–40, 50) and organization of the cortical cytoskeleton (50). In addition, Richardson *et al.* (59) have shown that co-expression of c-Src in cells expressing the dominant negative C-terminal domain of focal adhesion kinase can reconstitute cell spreading and motility and induces tyrosine phosphorylation of paxillin. Together, these observations raise the possibility that HGF-induced c-Src kinase activity may regulate cell motility through the cytoskeletal complex. This possibility is currently being investigated.

There is now growing evidence that the c-Src family protein-tyrosine kinases are involved in signal transduction pathways that result in cell growth, adhesion, and differentiation. c-Src kinase activity is required for cell proliferation induced by platelet-derived growth factor, colony stimulating factor-1, and epidermal growth factor (51, 60), and increased c-Src kinase activity is associated with many cancers. These observations support the notion that increased c-Src kinase activity in mammary carcinomas may play an important role in mammary tumor growth and development. Our findings involving transfection of a dominant negative c-Src kinase-defective mutant into SP1 cells represent the first direct demonstration of a requirement for c-Src kinase activity in HGF-induced cell mo-

tility and anchorage-independent growth of carcinoma cells, although interactions with other signaling molecules may also be involved. These data strongly suggest that HGF-induced association of c-Src kinase with Met and its activation are important in growth and transformation of mammary carcinomas and further argue that paracrine and autocrine HGF loops play a significant role in the transformed phenotype of some mammary carcinomas.

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ABSTRACT

Identification of paracrine and possible autocrine hepatocyte growth factor loops in breast and non-small cell lung carcinomas

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Hepatocyte growth factor (HGF) shows increased expression in human breast and non-small cell lung carcinomas (NSCLC), but its role in tumor progression is not clearly known. We have demonstrated co-expression of HGF and HGF receptor (Met) mRNA in invasive human breast carcinomas, as well as in regions of benign hyperplasia. Invasive breast carcinomas also showed reduced expression of E-cadherin. In contrast, most nonmalignant epithelia expressed Met and E-cadherin, but not HGF. These results suggest that establishment of an autocrine HGF loop promotes tumor growth and metastasis. We therefore examined HGF and Met expression in breast and NSCLC cell lines. Using semi-quantitative RT-PCR and western blotting, we showed that Met mRNA was expressed in all breast epithelial and carcinoma cell lines (7/7) tested, and all but one carcinoma cell line expressed detectable Met protein. Also, 8/8 NSCLC cell lines expressed Met mRNA, but only four cell lines expressed Met protein, implying incomplete translation or processing of Met in some carcinomas. In contrast, HGF mRNA and protein were expressed in 3/4 breast epithelial and carcinoma cell lines tested, and one of these cell lines showed active HGF in a Met phosphorylation assay. Also, 5/8 NSCLC cell lines expressed HGF mRNA and protein, and four of these showed active HGF. These findings suggest that: (a) *in vivo* tumor microenvironment may contribute to transcriptional and post-translational mechanisms that regulate HGF and Met expression and processing in carcinoma cells; and (b) both paracrine and autocrine stimulation by HGF in carcinoma cells may be important in invasive breast and NSCLC cancer. **(Supported by USAMRMC # 17-96-1-6251).**

c-SRC KINASE ACTIVITY IS REQUIRED FOR HGF-INDUCED MOTILITY AND ANCHORAGE-INDEPENDENT GROWTH OF MAMMARY CARCINOMA CELLS

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Over-expression and amplification of HGF receptor (Met) have been detected in many types of human cancers, suggesting a critical role for Met in growth and development of malignant cells. However, the molecular mechanism by which Met contributes to tumorigenesis is not well known. The tyrosine kinase c-Src has been implicated as a modulator of cell proliferation, spreading and migration; these functions are also regulated by Met. To explore whether c-Src kinase is involved in HGF-induced cell growth, a mouse mammary carcinoma cell line (SP1) which co-expresses HGF and Met, and a nonmalignant epithelial cell line (Mv1Lu) which expresses Met but not HGF were used. In this study, we have shown that c-Src kinase activity is constitutively elevated in SP1 cells and is induced in response to HGF in Mv1Lu cells. In addition, c-Src kinase associates with Met following stimulation with HGF. The enhanced activity of c-Src kinase also correlates with its ability to associate with Met. Expression of a dominant negative double mutant of c-Src (SRC-RF), lacking both kinase activity (K295R) and a regulatory tyrosine residue (Y527F), in SP1 cells significantly reduced c-Src kinase activity, and strongly blocked HGF-induced motility and colony growth in soft agar. In contrast, expression of the dominant negative c-Src mutant had no effect on HGF-induced cell proliferation on plastic. Taken together, our data strongly suggest that HGF-induced association of c-Src with Met and c-Src activation play a critical role in HGF-induced cell motility and anchorage-independent growth of mammary carcinomas, and further support the notion that the presence of paracrine and autocrine HGF loops contributes significantly to the transformed phenotype of carcinoma cells. (Supported by USAMRMC and CBCF).

c-SRC TYROSINE KINASE ACTIVITY IS REQUIRED FOR EXPRESSION OF HEPATOCYTE GROWTH FACTOR IN BREAST CARCINOMA CELLS

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Increased expression of hepatocyte growth factor (HGF) and its receptor, Met, has been identified as a possible independent indicator of recurrence in breast cancer patients. Our laboratory has previously shown increased expression of HGF and Met in regions of invasive human breast cancer (Tuck *et al.*, Am. J. Pathol. 144:675-682, 1996). In addition, we have found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met but not HGF. HGF also stimulates anchorage-independent survival of carcinoma cells. Together, these results suggest that establishment of an autocrine HGF loop in carcinoma cells may promote tumor progression. Increased activation of the tyrosine kinase c-Src also occurs in many cancer cells, and over-expression of c-Src induces mammary hyperplasia in transgenic mice. We have shown previously that c-Src kinase is activated in a mouse breast carcinoma cell line, SP1, which co-expresses HGF and Met, and c-Src kinase activity is required for HGF-induced motility and anchorage-independent growth of these cells (Rahimi *et al.*, J. Biol. Chem. 273:33714-21, 1998). To examine whether elevated c-Src kinase activity promotes the establishment of an HGF autocrine loop in breast carcinoma cells, we are studying the role of c-Src in regulating HGF mRNA and protein expression. The tyrosine kinase inhibitor herbimycin A causes a two-fold reduction in HGF mRNA in SP1 cells, while the phosphatidyl inositol (PI) 3-kinase inhibitor LY294002 shows no effect. In addition, expression of a dominant negative mutant of c-Src (K295R, Y527F) in SP1 cells leads to similar levels of reduction in HGF mRNA and functional protein. Transient expression studies in Cos-1 cells further support the notion that c-Src kinase activity is required for HGF transcription. Expression of an activated form of c-Src (Y527F) increases transcription from the HGF promoter, whereas expression of the dominant negative c-Src mutant significantly reduces HGF expression. Our results suggest that c-Src tyrosine kinase activity is required for transcriptional regulation of HGF expression and may be important in the establishment of an HGF autocrine loop in breast carcinoma cells. (Supported by USAMRMC Grant # 17-98-I-8330.)

CO-OPERATIVE INTERACTION BETWEEN HGF AND FIBRONECTIN IN ANCHORAGE-INDEPENDENT SURVIVAL AND GROWTH OF BREAST CARCINOMA CELLS: COMPLEMENTARY ROLES OF PHOSPHATIDYL-INOSITOL (PI) 3-KINASE AND C-SRC

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We previously showed that HGF and fibronectin (FN) promote anchorage-independent colony growth in agar of a murine mammary carcinoma cell line, SP1, which expresses both HGF and Met (Saulnier *et al.* ECR 222:360-9, 1996). In addition, formation of FN fibrils was required for colony growth. We now show that in the absence of anchorage, serum-deprived carcinoma cells exhibit reduced tyrosine-phosphorylation of Met and undergo cell death. Under these conditions, addition of exogenous HGF stimulates tyrosine-phosphorylation of Met and restores survival of carcinoma cells. Soluble FN also stimulates cell survival, and shows a co-operative survival response with HGF, but does not affect tyrosine-phosphorylation of Met in the absence of anchorage. Furthermore, inhibition of FN fibril formation does not affect HGF-, or FN-, induced cell survival. Together, these findings suggest that FN substratum promotes autocrine activation of Met, whereas soluble fibronectin most likely acts via a parallel pathway independent of Met in stimulating anchorage-independent cell survival. We have shown that inhibition of PI 3-kinase activity blocked HGF-, and FN-, induced anchorage-independent cell survival and colony growth, as well as HGF-induced DNA synthesis in adhering cells. In contrast, transfection of a dominant negative mutant form of c-Src (K295R, Y527F) strongly inhibited HGF-induced cell motility and anchorage-independent colony growth, but not DNA synthesis of adhering carcinoma cells. These findings show that a) HGF and FN stimulate a co-operative survival reponse in carcinoma cells, b) PI 3-kinase is a key downstream regulator of the HGF-, and FN-, induced cell survival effect, and c) c-Src is preferentially involved in cell motility and anchorage-independent growth. These findings suggest complementary signalling pathways regulate cell survival, DNA synthesis and moltility of breast carcinoma cells. (Supported by CBCF).